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The impact of bioactive agents PDGF & BMP on resolution of bony defects

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THESIS

THE IMPACT OF BIOACTIVE AGENTS PDGF & BMP ON RESOLUTION OF BONY DEFECTS

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DEDICATION

I will like to thank my father Mr. Kishan S. Tilwani for believing in me and for continuous support, guidance and unconditional love; I would not have been here without him. I will like to thank my sister Ms. Rashi Ahuja, my brother Mr. Kamlesh Tilwani for continuous encouragement and to see the potential in me… it would be a very long journey otherwise. I would like to thank my beloved wife Dr. Pooja Tilwani for her continuous support and tirelessly working with me to make this project a success.

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ABSTRACT

Bioactive agents are proteins that regulate cellular activities including cell migration, proliferation, differentiation and matrix synthesis. Over the last decades there has been a focused effort to understand how these agents influence repair or regeneration of bony defects. Platelet derived growth factor (PDGF) has potent chemotactic and angiogenic properties. Bone morphogenetic protein (BMP) is a known factor for osteoblasts. This study evaluated the impact of recombinant human PDGF and BMP-2 on resolution of critical bony defects (2 mm) using mouse calvarial bone cultures.

Calvaria from 5-7 day neonatal CD-1 mice were dissected and cultured in Dulbecco's Modified Eagle's Medium under sterile conditions. In the first experiment, two different delivery systems to deliver PDGF - freeze-dried bone allograft and beta- tricalcium phosphate were compared. The second experiment analyzed bone formation in response to BMP-2 in the presence or absence of freeze-dried bone allograft. The media was changed every 2 days and the spent media were analysed for calcium release. At the end of three weeks the calvaria were processed for histological observation, biochemical analyses and neutral red staining.

The results show higher bone formation in response to BMP-2 than PDGF. The presence of allograft inhibits this response. We found B-TCP to be a better delivery agent for PDGF compared to freeze-dried bone allograft. The histologic assessment showed development of new bone through intramembranous pathway that replicates native bone development in presence of BMP-2.

In conclusion our study proves that incorporation of two bioactive agents- PDGF and BMP-2 in an osteoconductive scaffold can induce repair and new bone formation in mouse calvarial bone cultures.

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LIST OF ABBREVIATIONS

aFGF ..... Acidic Fibroblast Growth Factor

ACS ..... Absorbable Collagen Sponge

ALP ..... Alkaline Phosphatase

bFGF ..... Basic Fibroblast Growth Factor

BMP ..... Bone Morphogenetic Protein

BSA ..... Bovine Serum Albumin

CGF ..... Cementum Derived Growth Factor

CM ..... Collagen Membrane

CT ..... Computed Tomography

CTG ..... Connective Tissue Graft

DBM ..... Demineralized Bone Matrix

DFDBA ..... Demineralized Freeze-Dried Bone Allograft

DMEM ..... Dulbecco's Modified Eagle's Medium

DNA ..... Deoxyribonucleic Acid

EGF ..... Epidermal Growth Factor

ePTFE ..... Expanded Polytetrafluoroethylene

FCS ..... Fetal Calf Serum

FDA ..... Food and Drug Administration

FDBA ..... Freeze-Dried Bone Allograft

GBR ..... Guided Bone Regeneration

GDF ..... Growth/Differentiation Factor

GEM-21 ..... Growth Factor-Enhanced Matrix-21

H & E ..... Hematoxylin and Eosin

HA ..... Hydroxyapatite

IGF ..... Insulin-Like Growth Factor

MDGF ..... Monocyte-Derived Growth Factors

NiTi ..... Nickel-Titanium

NR ..... Neutral Red

OP ..... Osteogenic Protein

PDGF ..... Platelet-Derived Growth Factor

PDL ..... Periodontal Ligament

PTHrP ..... Parathyroid Hormone-Related Protein

rhBMP ..... Recombinant Human Bone Morphogenetic Protein

rhPDGF ..... Recombinant Human Platelet-Derived Growth Factor

TCP ..... Tricalcium Phosphate

TGF-β ..... Transforming Growth Factor-beta

TNF-α ..... Tumor Necrosis Factor- alpha

TNF-β ..... Tumor Necrosis Factor- beta

VEGF ..... Vascular Endothelial Growth Factor

INTRODUCTION

Clinicians are frequently faced with the challenge of treating patients with significant alveolar bone loss due to periodontal disease, congenital abnormalities, tumors, traumatic injury/ or resorption secondary to tooth loss. Autogeneic, allogeneic and xenogeneic bone derivatives or synthetic biomaterials (bone substitutes) have been used as stand-alone therapies and in combinations to augment bone. Nonresorbable and bioresorbable barrier membranes usually expanded polytetrafluoroethylene (ePTFE) membranes or titanium mesh have also been used to contain implanted biomaterials. However, they must be removed in a second surgical procedure. Barrier membranes also have been used without bone biomaterials to support osteogenic (local) bone formation, a treatment known as guided bone regeneration (GBR). An increasing body of evidence suggests that synthetic bone biomaterials may delay or interfere with osteogenic bone formation because of slow resorption rates and may compromise the quality of bone. Thus, conventional reconstructive and grafting procedures may be ineffective in achieving bone regeneration leaving both the clinician and the patient dissatisfied with the outcome.

The need to develop alternative to bone grafting techniques led to investigation of the mechanism of bone formation and repair. Advances in cellular and molecular biology have led to better understanding of the intrinsic capacity of bone to regenerate during periods of homeostasis and following injury. This remarkable regenerative process is characterized by the remodeling cycle in which cells are recruited and differentiated for either bone formation or resorption. These activities are coordinated and regulated by an elaborate system of growth factors and cytokines.

Growth factors are natural biological mediators that regulate key cellular events that are part of the process of repair and regeneration. Binding of growth factors to specific cell membrane

receptors of target cells induce intracellular signaling pathways. These typically result in the activation of genes that change cellular activity and/or phenotype. These growth factors are either now available or in development for clinical application through recombinant DNA technology. In vitro and in vivo studies have confirmed that growth factors can enhance the capacity of tissues to regenerate by regulating cell chemo attraction, differentiation and proliferation.

The concept that endogenous substances from the human body are superior clinical therapeutic agents has established the need to define their clinical utility and efficacy. The use of growth factors to promote tissue regeneration represents a promising approach for periodontology and implantology.

Bone Formation

Bone is a specialized connective tissue with a mineralized matrix that functions to provide support, form and rigidity for the human skeleton.

Skeletogenesis in mammals requires coordinated activities of multiple cell types and consists of two distinct developmental processes.

1. Endochondral ossification – Most skeletal elements in the body, including all long bones, are derived through this process. Chondrocytes produce a cartilaginous template which is replaced with a mineralized matrix synthesized by osteoblasts.
2. Intramembranous ossification – Craniofacial bones are derived by condensed mesenchyme cells that directly differentiate into osteoblasts that produce an osteoid matrix and control mineralization.

Developmental Origin of Alveolar Bone

In human beings the process of skeletal patterning is completed within the first trimester of pregnancy. In mammals the mandibular and maxillary bones develop from the first branchial

arch during embryonic skeletal patterning. The alveolar bone and processes in the maxilla and mandible are formed by intramembranous ossification. The mandibular and maxillary alveolar processes houses and supports the dentition.

Types of Bone Defects

Classification of bony defects help dictate the type of grafting technique used for reconstruction. The simplest classification refers to the direction of bone loss. Horizontal bone loss indicates loss of bone width. Vertical bone loss is loss of height and is more difficult to reconstruct than horizontal loss.

Another classification scheme is based on the number of bony walls remaining at the graft site. Total vertical bone loss is a single walled defect and, due to the limited amount of remaining native bone, is the most challenging to reconstruct. A fresh extraction socket has five remaining walls (buccal, lingual, mesial, distal, and floor or apical bone) which provide native bone and simplifies graft containment.

Principles of Bone Grafting

Bone is classified as either cortical bone or trabecular bone. Cortical bone is made of dense, compact bone containing Haversian systems with lacunae housing osteocytes. Trabecular bone fills the marrow space between the cortices. This bone consists of trabeculae and is also known as spongy bone. Resident cells in the trabecular bone include osteoblasts, osteoclasts, and hematopoietic cells.

Bone Membranes

The cells responsible for the osteogenic potential of bone are housed in the tissues covering or lining bone. Both outer layer periosteum and internal endosteum contain osteoblasts and osteoprogenitor cells.

Bone Healing

It has been recognized from the time of Hippocrates (460– 370 BC) that of the many tissues in the human body bone has the highest potential for regeneration. When injured it heals by formation of new bone. In contrast most other tissues such as the heart muscle, voluntary muscles and the brain heal by replacement of the original tissue with connective tissue.

Bone healing can be classified as primary or secondary healing. Primary healing of bone implies direct contact or a gap of less than 1 mm between bone fragments. This process requires osteoclasts followed by osteoblasts secreting osteoid for future mineralization. Secondary bone healing occurs through formation of a callus of osteoid followed by mineralization and can be divided into three major phases (Figure 1). The inflammatory phase occurs immediately with formation of a hematoma which eventually becomes granulation tissue. The repair stage begins as inflammatory cells and fibroblasts invade the tissue and cause differentiation and recruitment of osteoblasts and provide a scaffold for further vascular ingrowth. The osteoblasts lay down osteoid and form the soft callus which is eventually ossified. The final stage involves remodeling that occurs over months to years and restores the bone to its original shape and close to its original strength.

The principles of primary and secondary bone healing can be applied to bone graft healing. The type of graft material used, block versus particulate, dictates which healing process occurs.

Cortical block bone grafts heal by a process similar to primary bone healing called creeping substitution. Once the nonvascularized graft material is transferred to the defect osteoclasts begin to resorb the graft material allowing for fibroblast ingrowth and the creation of a matrix for vascularization of the graft. The osteoclasts create voids in the graft material that are filled with osteoid produced by osteoblasts which mineralizes. The newly formed bone then undergoes remodeling and maturation. Ideally the grafted bone will be completely resorbed, and new bone will be formed. However the cortical block graft is never fully resorbed and replaced by new bone and necrotic centers remain mixed with the newly formed bone.

Particulate, cortical or cancellous bone grafts provide the necessary scaffold for ingrowth of osteoblasts and precursor cells into the defect and begin healing by apposition of bone. This apposition of bone is followed by resorption of the graft material. Ideally there is complete resorption of the graft material which is replaced by mature bone. Because cancellous grafts do not have to first undergo resorption before apposition they revascularize faster than cortical block grafts. There is a much higher percentage of newly formed bone and greater resorption of the graft material when particulate grafts are used.

Molecular Mechanism of Bone Healing

Autogenous cancellous marrow grafts undergo a well-documented and predictable healing process. Osteocompetent marrow stem cells and osteoblasts included in the graft survive at the grafted site through diffusion of oxygen and nutrients. During week 1 platelets attach, degranulate and release growth factors that are chemotactic, mitogenic, and angiogenic. These growth factors include: PDGFaa, PDGFbb, PDGFab, TGF-b1, TGF-b2, VEGF, EGF.

During weeks 2 and 3 the graft is undergoing revascularization through capillary ingrowth. As revascularization occurs the osteoblasts synthesize and secrete osteoid during weeks 2 through 8. As osteoid is secreted growth factors are released that stimulate osteoclastic activity leading to remodeling phase of bone healing lasting throughout the life of the bone. Approximately 90% of the grafted bone will be mature by 6 months.

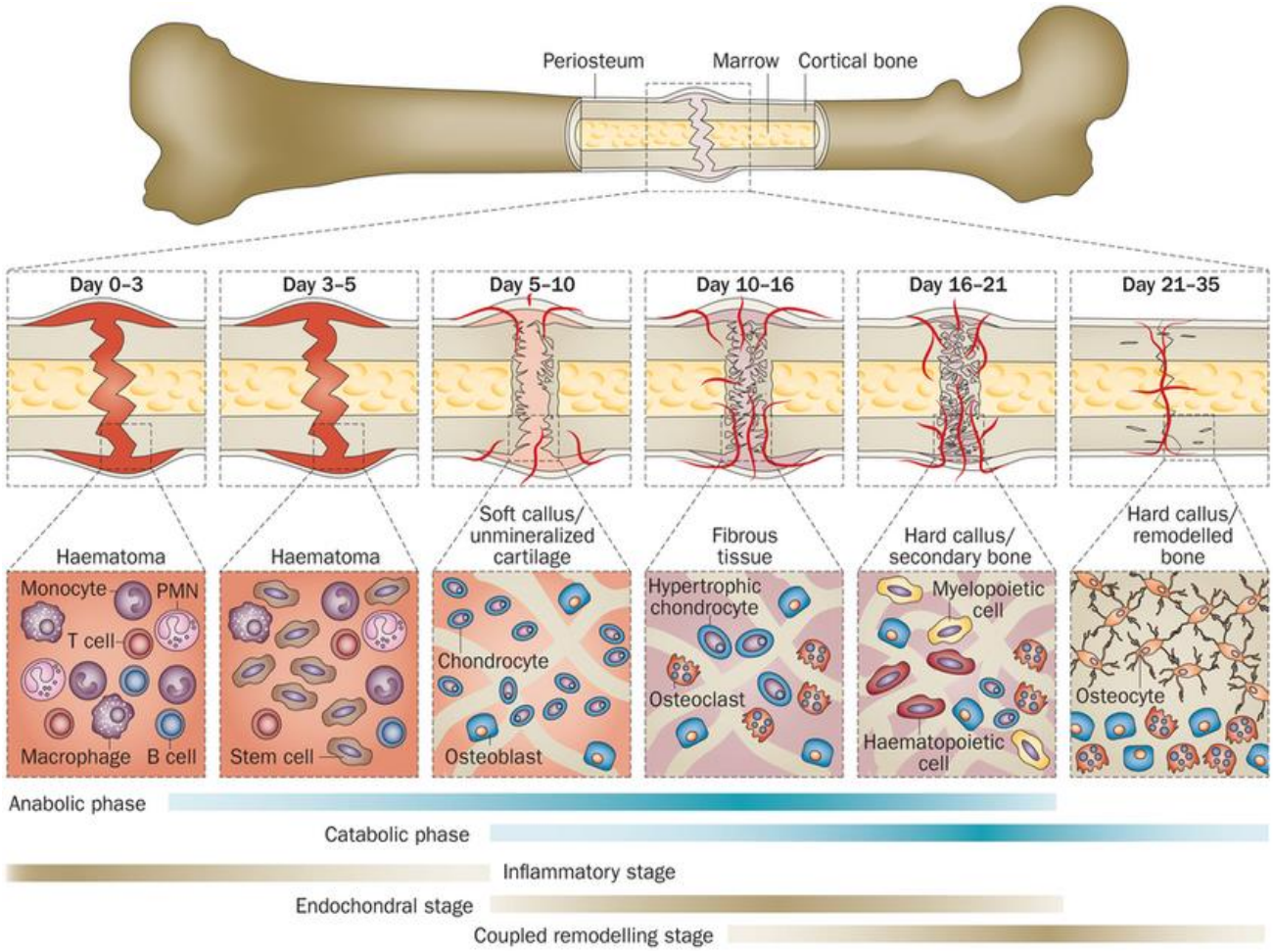


Figure 1: Stages of bone healing

The successful incorporation of bone grafts relies on many different factors. According to Misch (1993), these factors are:

1. Surgical asepsis
2. Soft tissue coverage
3. Graft immobilisation
4. Host site preparation
5. Host bone regeneration capacity
6. Optimisation of growth factors such as BMP

Surgical asepsis refers to the lack of acute infection. Infection within the bone results in pH of 2 and increases the risk of bone loss as grafts can dissolve in pH of 5.5 or less..

Tension-free soft tissue coverage helps maintain the graft by encouraging osteocompetent cell proliferation and healing by primary intention.

Graft mobilisation may result in fibrous encapsulation and non-union to the host bone. By ensuring stability of the graft the blood clot and associated growth factors can be maintained. Micromotion at the graft site cannot be avoided and may actually be beneficial to graft maturation by acting as a mechanical signal to stimulate graft healing. Excessive movement disturbs the blood supply and can create a sequestrum of the graft.

Revascularization from the host bone provides the graft with adequate growth factors and pluripotent perivascular cells that differentiate into osteoblasts. The blood supply for the grafted material initially comes from the adjacent native bone and the remaining soft tissues at the site. This is an important factor determining the success of the graft.

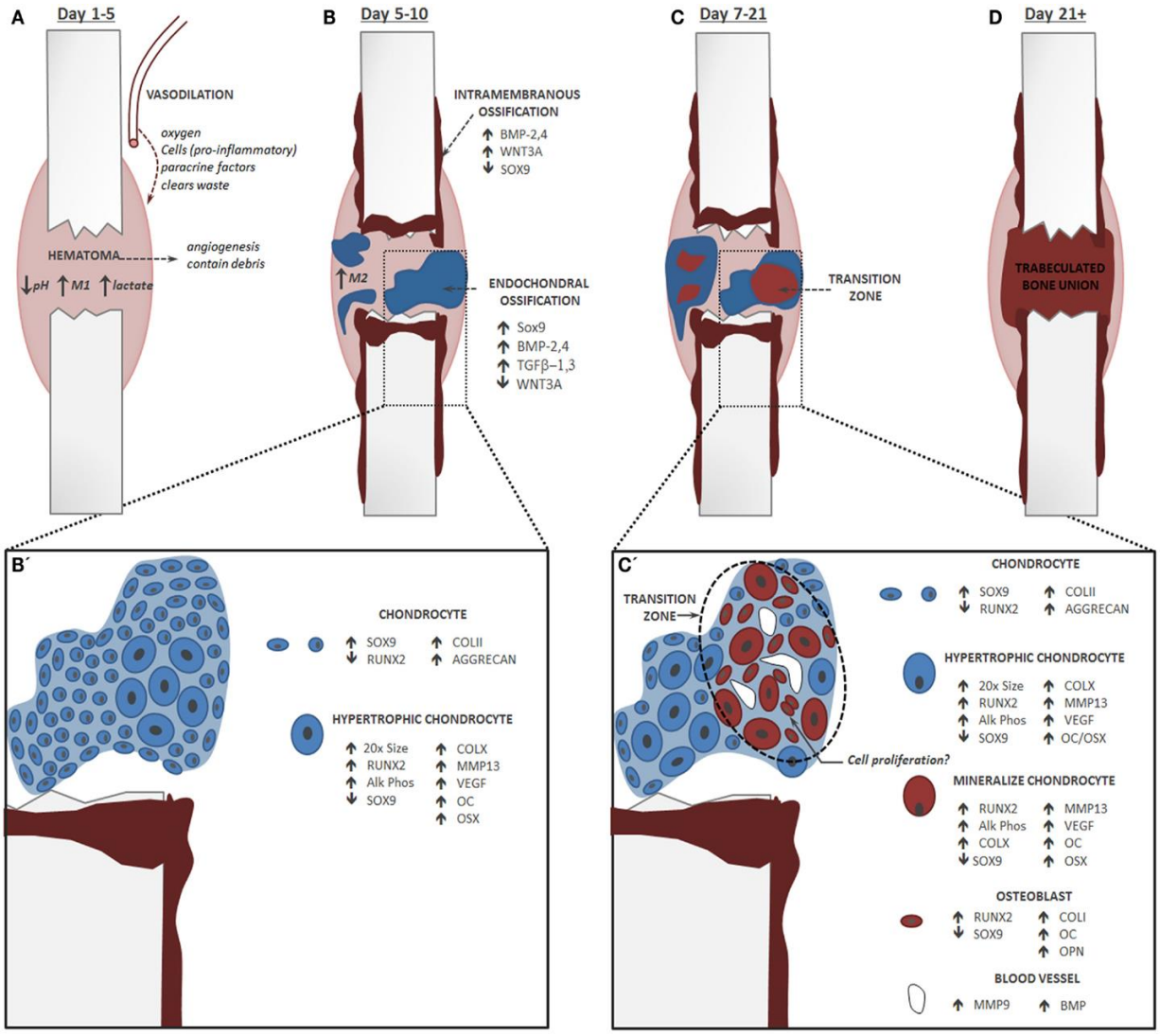


Figure 2: Molecular Mechanism of Bone Healing

Graft Function

Bell (1948) summarized the aim of bone grafting ‘‘to place a readily vascularizable osteogenic organic structure in intimate contact with a vascular osteogenic cancellous host bed’’ while adhering to ‘‘sound orthopedic principles”.

Bone grafting materials affect new bone formation at the defect site in many ways. The material can induce bone formation through cellular signaling or through the transfer of

osteocompetent cells or it may simply provide a scaffolding and have a space maintaining function for the host to grow new bone. Graft materials therefore can be classified based on their function and interaction with the host.

A graft that transfers osteocompetent cells to begin the bone forming process is called an osteogenic graft. The new bone at the site is formed from the cells transferred in the graft and not just from the osteocompetent cells at the defect site. The only osteogenic graft is an autogenous bone graft.

A graft that stimulates the host mesenchymal stem cells to differentiate and begin bone formation is called an osteoinductive graft. This process occurs through the transfer of proteins in the graft which begin a signaling cascade to tell the host to form bone.

A graft that simply provides scaffolding for the host to create new bone and has no biologic influence on the host is an osteoconductive graft. There are no proteins or cells present in the graft material to affect the host and influence bone formation.

Table 1: Characteristics of bone graft materials

Characteristic	**Material**
Osteogenesis	Autograft
Osteoinduction	BMP DFDBA DBM
Osteoconduction	Bio-Oss Calcium phosphates Calcium sulfate Collagen FDBA Glass ionomers HA NiTi

Classification of Graft Types

Bone grafting materials can be classified using a combination of source of origin and mineral content. The source can be subclassified further by species and by location. Cortical bone is a graft taken from the outer compact bone and can be in a particulate or block form. Cancellous graft material is taken from the softer trabecular bone. It is usually found in a particulate form but with proper processing it also can be offered in block form. Block bone grafts are typically cortical in nature. They hold their form very well and can be shaped and formed.

Block bone can be from multiple sources such as allografts, xenografts, and synthetic block grafts. Multiple autogenous intraoral and extraoral sites can be used to harvest block grafts. Particulate grafts are able to withstand exposure to the oral cavity environment better than block

grafts. Particulate grafts are available from multiple biologic and synthetic sources. These grafts can be cortical, cancellous, or a cortical and cancellous mixture.

Another method of classifying grafts is based on their source. The gold standard in bone grafting material is autogenous bone, harvested from the subject who is receiving the graft. There is no antigenic response to the grafted material and no graft rejection. Autogenous cancellous bone grafts produce the most successful and predictable results. There are essentially two forms of nonvascularized free autogenous bone grafts: cortical and cancellous. Cancellous grafts are revascularized more rapidly and completely than cortical grafts. Autogenous grafts provide all three functional properties of a graft material: osteogenesis, osteoinduction, and osteoconduction.

The allograft also known as a homologous graft is non-vital osseous tissue taken from a genetically similar donor. There are three forms of allogeneic bone: fresh frozen, freeze-dried and demineralized bone matrix (DBM). The most common source of allograft bone is cadaver donors. These grafts are processed to reduce antigenicity and all cells are killed. Therefore there is no transfer of osteocompetent cells with this graft type. Allografts provide the graft functions of osteoinduction and osteoconduction.

Xenografts also known as heterografts are from genetically dissimilar, nonhuman sources. Common sources are bovine and porcine animals. It has the highest antigenic potential because it is from a nonhuman source. Xenografts provide the graft functions of osteoinduction and osteoconduction.

Synthetic graft materials or alloplastic bone substitutes are synthetic substances that have been processed for clinical use in osseous regeneration . These grafts are available in block and particulate forms. There is no cellular or protein material with these grafts. Their functional

classification is only osteoconduction. These include hydroxyapatite, tricalcium phosphate, bioglass and calcium sulphate.

Table 2: Graft classification based on source

Source	Characteristics
Autograft	Taken from the host; The gold standard in bone grafting The only graft source that is osteogenic
Allograft	Graft taken from a genetically similar donor Cadaver graft
Xenograft	Graft taken from a genetically dissimilar donor Most commonly bovine or porcine source
Synthetic Graft	Graft not taken from a living donor No cellular or protein products in this graft

Polypeptide Growth Factors

Biologic mediators regulate cellular activities, including cell migration, proliferation, differentiation, and matrix synthesis. Over the last decades there has been a focused effort to understand how polypeptide growth factors influence repair or regeneration of tissues. These native ligands have pleiotropic effects that support regeneration and accelerate healing. They exert their effects by binding to specific cell membrane receptors and initiate complex cascades eventually reaching a nuclear target gene to generate signals for specific phenotype expression. The factors that have the ability to stimulate deposition of bone are known as osteoactive agents, osteoinducers, osteopromotors or bioactive peptides. The phenomenon of osteoinduction was first described by Urist and co-workers in 1952. Examples of polypeptide growth factors in bone, cementum, and healing tissues include platelet-derived growth factor (PDGF), vascular endothelial

growth factor (VGF), transforming growth factor-a and -b (TGF-a, TGF-b), epidermal growth factor (EGF), insulin like growth factors-I and -II (IGF-I, IGF-II), cementum derived growth factor (CGF), parathyroid hormone-related protein (PTHrP), bone morphogenetic proteins (BMPs), tumor necrosis factor (TNF), monocyte-derived growth factors (MDGF), and acidic and basic fibroblast growth factor (aFGF, bFGF).

While growth factor proteins are potent stimulators of wound repair the utilization of these proteins contained within blood platelets for oral surgical treatment did not occur until 1998 when Marx and co-workers proposed the use of autologous platelet concentrates. Platelet concentrates require isolating platelets present in autologous blood by a selective centrifugation and subsequently activating them to release their growth factor content. These include super-physiologic concentrations of PDGF, TGF-β and IGF-I among others. These factors are applied to the treatment site to promote tissue regeneration or repair.

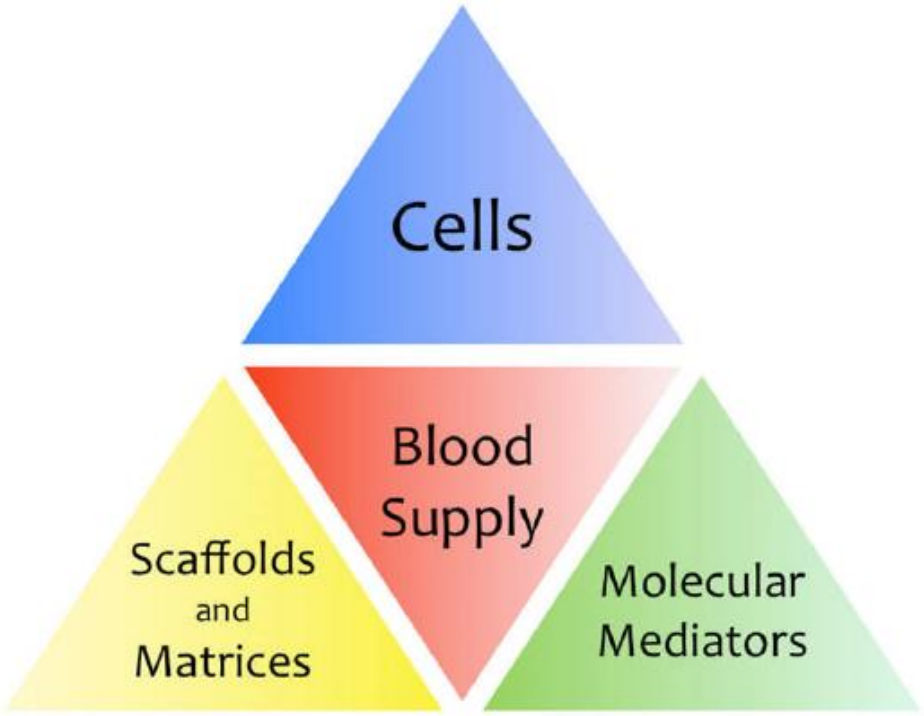


Figure 3: Four basic elements are required for repair and regeneration: 1) adequate blood supply and wound stability, 2) a source of bone and ligament forming cells, 3) a supporting scaffold or matrix, and 4) growth factors to regulate cell migration, proliferation and synthesis and angiogenesis for revascularization of the site.

This study was designed to determine the effect of two growth factors- PDGF and BMP-2 on osteoblast differentiation, multiplication and bone formation. Freeze dried bone allograft was used in conjunction with these growth factors as a scaffold and to promote bone induction.

Bone Morphogenetic Protein

History of Bone Morphogenetic Protein

In 1889 Senn noticed that decalcified bone could induce healing of bone defects. He treated osteomyelitic defects in bone by using decalcified ox bone with iodoform. However, his findings were not easily reproduced. In the 1930 Levander noted that crude alcohol extracts of bone induced

new bone formation when injected into muscle tissue. In 1961 Sharrard and Collins reported the use of ethylene diamine tetra acetic acid–decalcified allograft bone for spinal fusion in children. This study was supported by reports by Ray and Holloway.

The seminal discovery of the ability of the bone matrix to induce new bone was made by Urist in 1965. He showed that decacified bone extracts induced new bone in a muscle pouch in a rat model. He coined the term "bone morphogenetic protein" or "osteogenic protein" for the active ingredient in this extract. His research was hampered by the fact that there was no reproducible assay for the protein. Reddi and Sampath (1983) advanced the field when they developed a crude but highly reproducible assay for ectopic bone formation.

The first clinical study was conducted in 1988 by Johnson et al using purified human BMP. Two groups at Creative BioMolecules and Genetics Institute simultaneously sequenced the gene for various BMPs which resulted in a patent dispute that was subsequently resolved. Human BMPs are now produced by recombinant DNA technology to yield a protein free from the risk of infection or allergic reaction.

The final landmark in this saga is FDA approval in 2002 for OP-1 (BMP-7) for long bone defects (Stryker Corp., Kalamazoo, MI) and BMP-2 in a collagen carrier for anterior lumbar interbody fusions (Medtronic Sofamor Danek, Memphis, TN).

Classification and Chemical Structure of Bone Morphogenetic Protein

Bone morphogenic proteins are members of the TGF-β superfamily of growth factors. TGF-β was named because of its ability to transform cultured fibroblasts into their activated form. Like all members of the TGF-β family BMPs are synthesized as precursor proteins that contain a hydrophobic leader sequence. The active protein is in the carboxy terminal end of the precursor

molecule and contains seven cysteine residues in identical positions in all members of the TGF-β superfamily. In addition BMPs contain N-linked glycosylation sites.

BMP proteins can be broadly classified into three subfamilies. BMP-2 and BMP-4 have 80% amino acid sequence homology. The second group consisting of BMP-5, -6 and -7 have 78% amino acid sequence homology. BMP-3 is different from other members of the BMP family and stands alone. The mature segment of the BMPs contains seven cysteine amino acid residues. Six of these residues form intrachain disulphide bonds that produce a rigid "cysteine-knot" molecular structure. The seventh cysteine residue forms dimers via an interchain disulphide bonds. The dimers may be either homo- or heterodimers. Formation of homo/heterodimers increases the variability of the effector molecule. The reason for this redundancy is not fully understood but probably offers a larger repertoire of molecules with similar functions. Differences between members of the TGF-β superfamily are in the varying turns, α-helices and β-sheets as documented by the analyses of crystal or solution structures for TGF-β1, TGF-β2, BMP-7, or BMP-2 (Griffith et al. 1996; Hinck et al. 1996; Scheufler et al. 1999; Schlunegger et al. 1992).

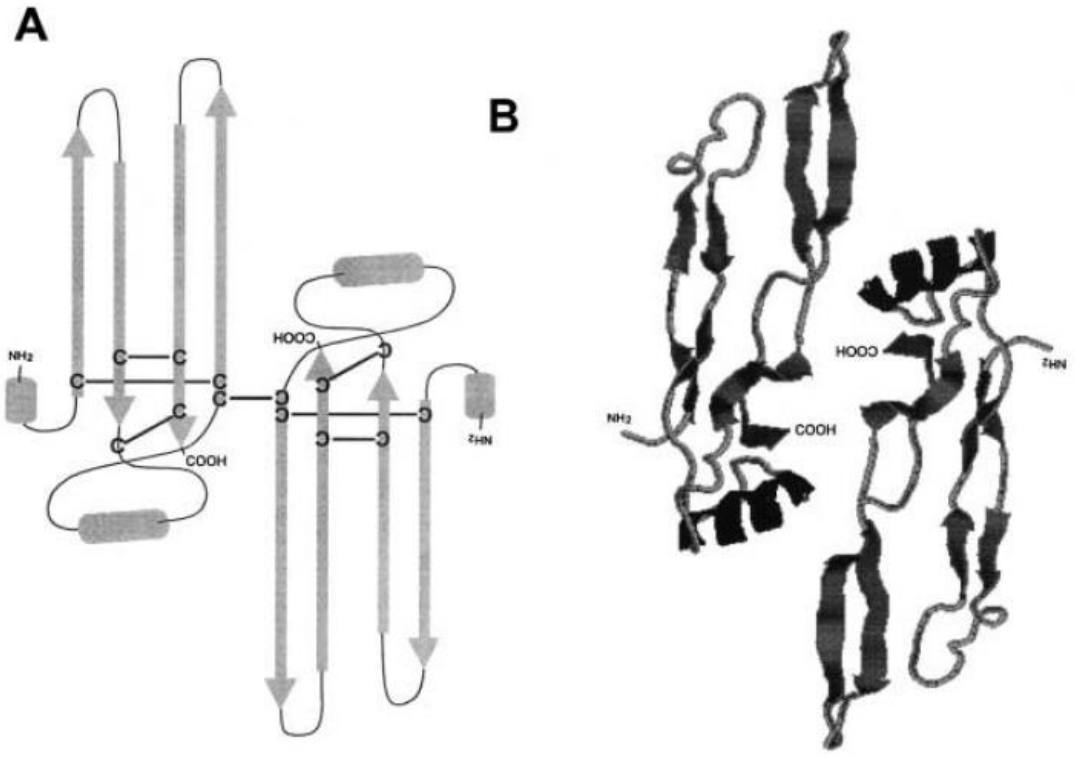


Figure 4: A, B - Structure of bone morphogenetic protein (*BMP*). The active molecule is a dimer in which seven to nine cysteine residues are involved in the formation of disulfide bonds to build a so-called cysteine-knot.

BMP is unique among the growth factors. It is not only a mitogen stimulating the multiplication of connective tissue cells but is the only morphogen that has the ability to transform connective tissue cells into osteoprogenitor cells. All other growth factors such as TGFβ, IGF, FGF, PDGF and VEGF induce multiplication of cells but do not transform one cell type into another.

Naturally occurring BMP, collected through purification of demineralized bone matrix, is available in minute quantities. Using recombinant DNA technology human homologs of bovine BMP coding sequences were acquired and mammalian cells were engineered to express each

protein in a purified form. Therefore using recombinant DNA technology a large, uniform supply of recombinant human BMPs, rhBMP-2 and rhBMP-7 are commercially available.

Table 3: Bone morphogenetic protein family (Hogan 1996; Kingsley 1994)

BMP	**Function**
BMP-2	osteoinductive, osteoblast differentiation, apotosis
BMP-3	(osteogenin) most abundant BMP in bone, inhibits osteogenesis BMPs
BMP-4	osteoinductive, lung & eye development
BMP-5	Chondrogenesis
BMP-6	osteoblast differentiation, chondrogenesis
BMP-7(OP-1)	osteoinductive, development of kidney & eye
BMP-8 (OP-1)	Osteoinductive
BMP-9	nervous system, hepatic reticuloendothelial system, hepatogenesis
BMP-10	cardiac development
BMP-11(GDF-8)	patterning mesodermal & neuronal tissues myostatin
BMP-12(GDF-7)	induces tendon-iliac tissue formation
BMP-13(GDF-6)	induces tendon & ligament-like tissue formation
BMP-14(GDF-5)	chondrogenesis, enhances tendon healing & bone formation
BMP-15	modifies follicle-stimulating hormone activity

* GDF = growth/differentiation factor

*OP = osteogenic protein

Biologic Activity of Bone Morphogenetic Protein

BMPs stimulate a sequence of events typical of endochondral bone formation: recruitment of mesenchymal cells, differentiation to chondrocytes, chondrocyte hypertrophy, calcification of cartilage matrix, osteoblast differentiation and bone formation, and eventual remodeling of newly formed bone and marrow creation (Figure 5). The quality of BMP-induced bone is indistinguishable from natural bone. Bone healing under normal conditions in the absence of BMP occurs from the bone margins of the gap to be filled and progressively creeps from the ends toward the center whereas BMP-induced bone forms concurrently throughout the defect. Thus, BMP transforms inflammatory cells into osteoprogenitor cells that may freely cross the gap and lay down bone to close the gap. Bone induced by BMP matures faster than bone healed naturally. In human beings healing is complete by 8 to 10 weeks as opposed to 12 to 16 weeks when an autograft is used.

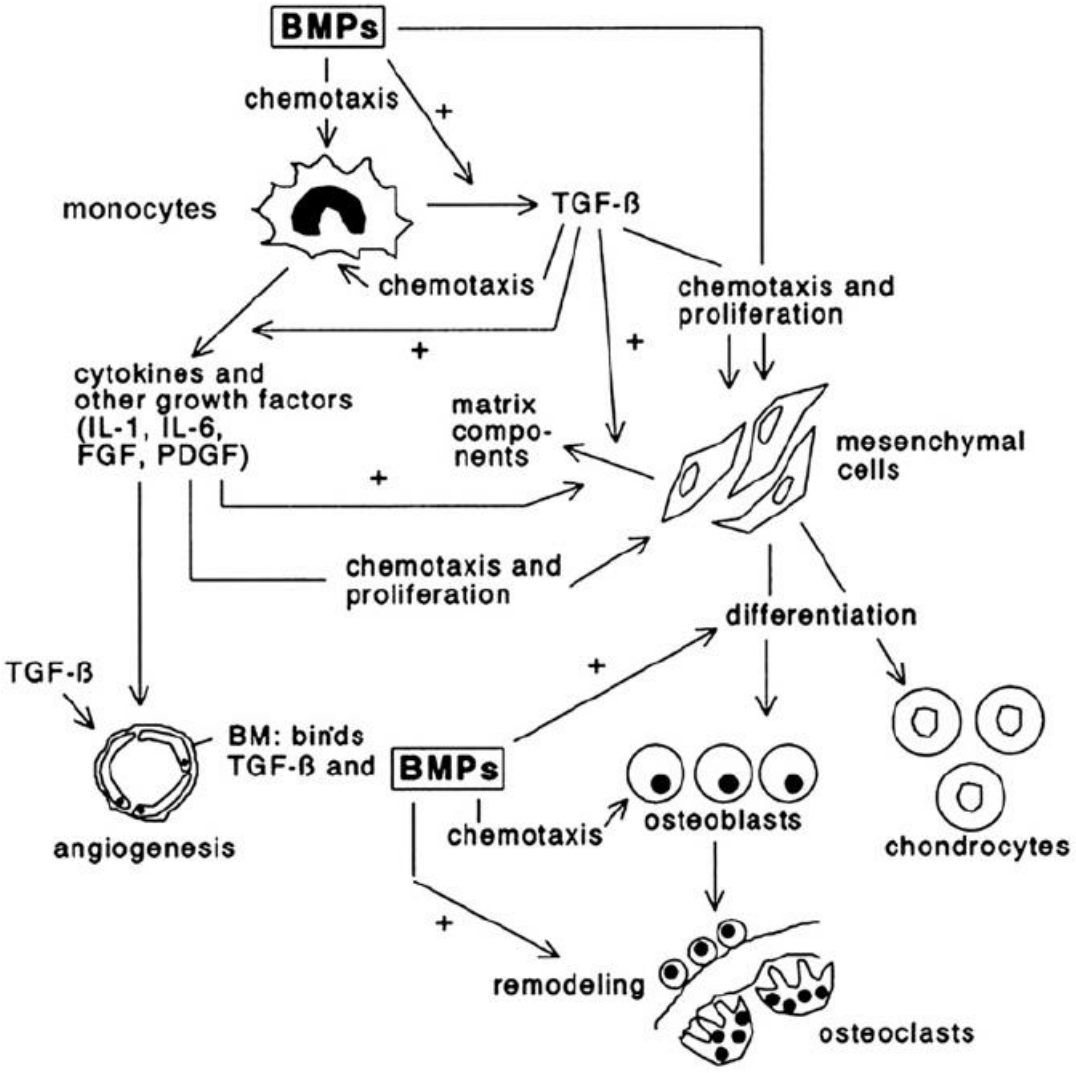


Figure 5: Mechanism of action of BMPs in bone repair. Sequence of events observed in endochondral bone formation induced by BMPs: recruitment and proliferation of monocytes and mesenchymal cells, differentiation into chondrocytes, calcification of the cartilage matrix, vascular invasion with associated osteoblast differentiation and bone formation, and remodeling of the newly formed bone. (Reproduced from Termaat MF et al, 2005)

Dosage of Bone Morphogenetic Protein

Osteoinductivity of individual BMPs has a steep dose-response curve. Low doses result in little cartilage and bone formation and increasing concentrations eventually result in direct (intramembranous) ossification. The concentration of the BMP at the site of implantation is more crucial than the total dose of BMP administered. The suggested human therapeutic doses (0.88 mg/mL of sterile water for rhBMP-7 and 1.50 mg/mL of sterile water for rhBMP-2) were derived from nonhuman primate studies and verified in clinical orthopedic studies. The doses of rhBMP required to induce bone formation in human beings is higher than endogenous concentrations of BMPs (0.002 mg of BMP per kilogram of pulverized bone). Requirements for supraphysiologic doses may be secondary to the tightly regulated signaling pathways and due to brisk local and systemic clearance of BMP. The potency of multiple resident BMPs may be superior to singly applied rhBMP. Dose response studies in animal models have indicated that the supraphysiological dose approaching 3 to 3.5 mg of BMP is sufficient in virtually all cases to induce new bone and to bridge the osseous defect. Additional doses of BMP do not confer any benefit in terms of fusion rate or time for the fusion to occur.

Carriers for Bone Morphogenetic Protein

Hollinger and colleagues(2008) reported that less than 5% of BMP remains at the implantation site when used alone but combining BMP with gelatin foam, collagen,or calcium phosphate pastes increases the retention by 5% to 15%. Ideally a matrix allows a predictable rate of release of the factor. A burst release of rhBMP to first recruit mesenchymal cells to the site of implantation followed by sustained release to differentiate the osteoprogenitor cells toward osteoblast phenotype is preferred kinetics. The delivery system serves as a scaffold with pores

large enough (>100 mm) to allow cellular infiltration and vascular ingrowth. The scaffold also provides a template onto which initial bone deposition can occur.

Carrier materials can be classified into four major subgroups: natural polymers, inorganic materials, synthetic polymers, and composites. Although BMP has been combined

with matrices from all of these categories in various preclinical and clinical studies, there are currently only three carriers approved for clinical use. The approved carriers include type 1 absorbable collagen sponge (ACS), particulate bone-derived type 1 collagen matrix, and a combination of BMP-7 delivered in the particulate collagen matrix combined with carboxymethyl cellulose.

An absorbable collagen sponge (ACS) was the first FDA-approved BMP carrier. ACS is a bovine type I collagen matrix that is soak-loaded with a BMP solution before surgical implantation. They have received the most attention because of its historical safety profile, excellent biocompatibility, degradation into physiologic products, intermediate BMP release rate kinetics, and positive effects on wound healing.

Clinical Efficacy and Safety

In preclinical and clinical studies BMP has a positive safety profile. With the use of supraphysiologic doses of BMP required to promote bone formation no harmful systemic or direct toxic effects attributable to BMP have been reported. Human studies using rhBMP-2 for alveolar ridge augmentation, maxillary sinus augmentation, and spinal fusion have exposed no harmful consequences. When rhBMP-2 is implanted with ACS the slow release results in minimal levels rhBMP-2 detectable in the systemic circulation and that which enters the circulation is rapidly cleared. Similarly clinical use of rhBMP-7 in long bone defects, spine fusion, and thoracolumbar

fusions have revealed no adverse systemic events. Clinical studies and case reports have described greater local edema at rhBMP implant sites versus controls in the postoperative period. The increased edema is thought to be secondary to recruitment and influx of mesenchymal cells by the rhBMP grafts.

Immunogenicity of rhBMP-2 is reportedly low but may be higher when combined with ACS carriers. Friedlaender and colleagues (2001) found 10% of patients treated with rhBMP-7 for tibial nonunion developed antibodies to the protein but titers were low and transient and no harmful outcomes were reported.

Currently two rhBMPs with coupled delivery systems have been granted Food and Drug Administration (FDA) approval. OP-1 (Stryker Biotech, Hopkinton, Massachusetts) contains rhBMP-7 plus bovine collagen and is reconstituted with saline to create a paste. The INFUSE(Medtronic Sofamor Danek USA, Memphis, Tennessee) graft includes freeze-dried rhBMP-2 that is reconstituted with saline and then injected onto an absorbable collagen sponge.

The FDA approved INFUSE for use in sinus augmentation and alveolar ridge augmentation associated with extraction sockets in March 2007. The approval for maxillofacial applications was based on five clinical studies that included 312 patients. Cochran and colleagues (2000) reported on 12 patients who received rhBMP-2/ACS at 0.43 mg/mL in extraction sockets or alveolar ridge augmentation. They were treated with endosseous implants in the augmented areas and bone core samples of the grafted region were obtained for histologic analysis. Three-year follow-up evaluation found that all implants were stable and without clinical or radiographic complications and bone biopsies showed normal bone formation. The investigators concluded that rhBMP-2/ACS could be safely used in extraction sites and for localized alveolar ridge augmentation. The pivotal study supporting use of rhBMP-2/ACS for ridge preservation was a randomized, masked,

placebo-controlled, multicenter clinical study reported by Fiorellini and colleagues (2005), which looked at two sequential cohorts of 40 patients with a total of 95 defects. The patients required localized ridge augmentation/preservation of maxillary teeth (premolar and anterior) with greater than or equal to 50% buccal bone loss in the extraction socket. Patients were randomized to receive 0.75 mg/mL or 1.50 mg/mL rhBMP-2/ACS, placebo (ACS alone) or no treatment in a 2:1:1 ratio. Surgical procedures included the following: full-thickness periosteal flaps, extraction of teeth, perforation of socket walls with round bur, placement of placebo or rhBMP-2 as strips into the socket and a larger piece placed over the entire site, and a tension-free flap closure. Efficacy parameters included CT-based measurement of alveolar bone height and width of various levels of the extraction socket at baseline and 4 months after treatment and, secondarily, whether or not adequate bone volume was attained to place dental implants. In addition, bone densities of native bone and treatment areas were calculated with reference to a standard density block. Sockets treated with 1.5-mg/mL rhBMP-2/ACS achieved significantly greater bone augmentation versus controls. Sufficient bone volume for dental implant placement occured twice as often in the rhBMP-2 groups compared to placebo and no treatment sites.

Boyne (1997) implanted RhBMP-2/ACS into the maxillary sinuses of 12 patients with inadequate bone height in the posterior maxilla. In 11 patients available for follow-up evaluation mean bone height achieved after grafting was 8.51 mm and there were no serious immunologic or adverse events associated with the procedure. In a follow-up phase II study Boyne and colleagues (2005) assessed the safety and efficacy of two concentrations (0.75 mg/mL and 1.50 mg/mL) of rhBMP- 2 used in human maxillary sinus augmentation to induce sufficient bone for implant placement. They also examined the success rate of implants placed in the induced bone after 36 months of function. These results were compared with maxillary sinuses augmented with bone

graft and subsequent implants placed in these sites. The mean increase in alveolar ridge height at 4 months post surgery was comparable between the groups (ranging 9.5 mm to 11.3 mm). Bone density was significantly greater after the first 4 months in the bone graft cohort and greater in the 1.50-mg/mL group than the 0.75-mg/mL patient. Bone density equalized in the treatment groups after 6 months of implant functional loading. Overall, implant survival rates after 36 months of functional loading were 81%, 88%, and 79% for the bone graft, 0.75-mg/mL, and 1.50-mg/mL treatment groups, respectively. The investigators concluded that both concentrations of rhBMP-2 are safe for maxillary augmentation procedures and induce a similar amount of bone formation as does a bone graft. The higher concentration of rhBMP-2 induced bone formation at a faster rate than the lower concentration.

BMP-2 use has also been considered in grafting periodontal defects, peri-implant defects, supra-alveolar ridge defects, and peri-implantitis–induced bone loss.

Platelet Derived Growth Factor

PDGF is a naturally occurring protein contained in alpha granules of platelets and locally released during clotting following soft or hard tissue injury. The original source of PDGF was platelets but PDGF or PDGF-like peptides have been isolated from a variety of normal and neoplastic tissues including bone matrix and osteosarcoma cells. Once it is released from the platelets PDGF binds to specific cell surface receptors promoting rapid cell migration (chemotaxis), and proliferation (mitogenesis) in the area of injury. In vitro and in vivo studies have demonstrated that PDGF is a potent chemotactic and mitogenic factor for gingival and periodontal ligament fibroblasts, cementoblasts and osteoblasts. PDGF stimulates bone DNA and protein synthesis and may be a systemic or local regulator of skeletal growth. As a systemic growth factor

it is released during platelet aggregation and has important effects in the early stages of fracture healing. As a local factor it interacts with other hormones and growth factors to promote bone cells to respond to other factors present in the skeletal tissue. In addition to its effects on bone formation PDGF stimulates bone resorption thus has complex effects on bone remodeling.

Recombinant DNA human platelet-derived growth factor (rhPDGF) was the first recombinant protein to be approved by the US Food and Drug Administration for treatment of chronic foot ulcers in diabetic patients (Regranex, Ethicon Inc. Somerville, NJ) and in the repair of periodontal bone defects as GEM 21S® (Osteohealth®). GEM, a growth factor-enhanced matrix, is delivered at a dosage of 0.3 mg/ml and is packaged with an alloplastic carrier of beta-tricalcium phosphate (β-TCP) graft particulate. Widespread use has established the safety and effectiveness of PDGF for soft tissue regeneration. Additionally rhPDGF for bone regeneration has been rigorously tested in preclinical studies which indicate that PDGF has the potential to direct and control bone regeneration in human beings.

Structure of PDGF

Four different chains (A, B, C, and D) have been identified in the structure of PDGF. PDGF is a family of five heterodimeric and homodimeric proteins (PDGF-AB, PDGF-AA, PDGFBB,PDGF-CC, and PDGF-DD), with PDGF-BB being the most effective. It stimulates human PDL cell proliferation and collagen synthesis in a time- and dose-dependent manner with maximum effect at 24 hours at a dose of 10 ng/mL. A- and B-chains of PDGF contain 100 amino acid residues and have 60% amino acid sequence identity. Each chain has 8 cysteine residues which are conserved between the 2 chains; 2 of the cysteine residues are involved in cysteine bonds between the 2 subunits in the PDGF dimer and the other 6 are engaged in intrachain disulfide

bonds. Mutation of the interchain disulfide bonds is compatible with retained biological activity of PDGF because the molecule is still a dimer.

The A, B, C, and D chain genes of PDGF are localized to the chromosomes 7p22, 22q13, 4q31, and 11q22 respectively. Their expression is independently regulated by PDGF receptors (PDGFRs). PDGF isoforms exert their cellular effects by activating two structurally related cell surface receptor tyrosine kinases (a-PDGFR and b-PDGFR).The a-PDGFR and b-PDGFR genes are localized on chromosomes 4q12 and 5q33, respectively.

Physical and Chemical Characterizations of PDGF

PDGF is difficult to purify as it is in very small quantities in platelets that have proteolytic activity. PDGF is a basic glycoprotein with pI of 10.2. PDGF-A is 31 kD and contains 7% carbohydrate whereas PDGF-B is 28 kD and contains 4% carbohydrate. PDGF A and B have essentially equal mitogenic activity and similar amino acid composition and immunological reactivity.

The response to PDGF depends on the isoforms of PDGF, the type of target cell, and the specific cell-surface receptor expressed on the target cell. At a wound site PDGF attracts neutrophils and macrophages and stimulates macrophages to release additional growth factors that are important for wound healing. PDGF receptors are found on all of the connective-tissue cells associated with bone healing, including fibroblasts, vascular smooth-muscle cells, osteoblasts and chondrocytes.

PDGF isoforms exert their effects on target cells by activating two structurally related protein tyrosine kinase enzymes. The α- and β-receptors have molecular weights of 170 and 180

kD respectively after attachment of their carbohydrates. The structures of PDGF receptors are similar to the colony stimulating factor-1 receptor and the stem cell factor receptor.

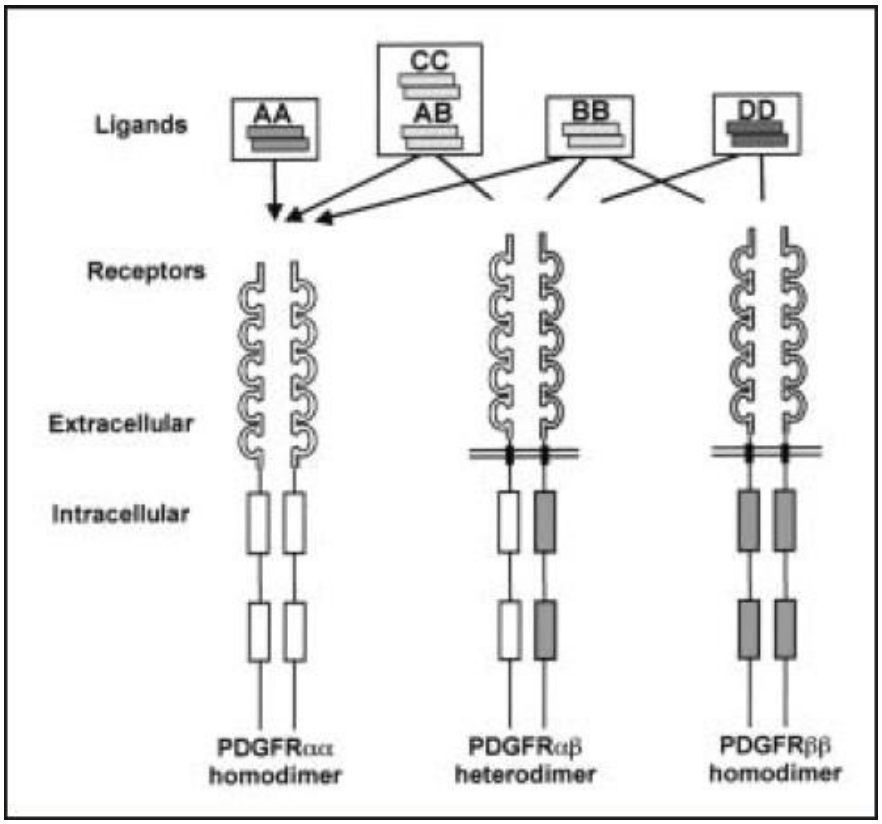


Figure 6: Five isoforms of PDGF ligands and their specificity of binding to α and β receptors (Alvarez RH et al. 2006)

Cellular Effects and Expression of PDGF Receptors

The α-receptor binds both the A- and B-chains of PDGF with high affinity whereas the β-receptor binds only the B-chain with high affinity. Both α and β-receptor homodimers transduce mitogenic signals. Activation of the β-receptor stimulates chemotaxis; in contrast, activation of the α-receptors inhibits chemotaxis of certain cell types including fibroblasts and smooth muscle cells. Both the receptors mediate an increase in intracellular $Ca2^{+}$ concentration albeit the β-receptor is

more efficient than the α-receptor. PDGF also inhibits gap junctional communication between cells and exerts an antiapoptotic effect. The response of a cell to PDGF stimulation will depend on the ratios of α- and β-receptors on the cell. The classical target cells for PDGF express both receptors but generally higher levels of β-receptors.

Table 4: Types of cells that express PDGF receptors (Alvarez RH et al. 2006)

Cell type	**PDGF-α receptor**	**PDGF-β receptor**
Platelets and Megakaryocytes	+	NA
Fibroblasts	+	+
Pericytes	+	+
Vascular smooth muscle cells	+	+
Neurons	+	+
Mammary epithelial cells	NA	+
Myeloid hematopoietic cells	NA	+
Macrophages	NA	+
Kidney mesangial cells	+	+
Ito liver cells	NA	+
Myoblasts	+	NA

PDGF in Bone Regeneration and Bone Grafting

PDGF is present in platelets and produced by macrophages and increases the mesenchymal cells in a wound. This is accomplished by two activities:

(1) as platelets aggregate in the wound they release PDGF which diffuses into the surrounding tissue and acts as a chemo attractant to recruit cells into the wound

(2) stimulates proliferation of cells and increases deposition of matrix.

PDGF activates cell membrane receptors on target cells which activate casacade of reactions that result in high-energy phosphate bonds on internal cytoplasmic signal proteins. The bonds then activate signal proteins to initiate specific activities within the target cell. The specific activities of PDGF include mitogenesis (increase in the cell populations of healing cells), angiogenesis (endothelial mitoses to produce functioning capillaries), and macrophage activation (debridement of the wound site and a second phase source of growth factors for continued repair and bone regeneration).The role of PDGF and mechanism of action in bone regeneration is illustrated in Figure: 7.

The life span of a platelet in a wound and the period of direct influence of its growth factors are less than 5 days. The extension of healing and bone regeneration activity is accomplished by two mechanisms. First, it increases the population of marrow stem cells and activates them to dfferentiate into osteoblasts that secrete TGF-β. The second and more important mechanism is chemotaxis and activation of macrophages that replace platelets as the primary source of growth factors after the third day. The macrophage is attracted to the graft by the actions of PDGF and by an oxygen gradient between the graft dead space and the adjacent normal tissue.

All platelets degranulate within 3-5 days and their initial growth factor activity may expire by 7-10 days. Administration of PDGF appears to ‘‘jump-start’’ the beginning of a cascade of regenerative events that continue to form a mature graft. Bone regeneration begins by the initiation of mitosis in stem cells and endothelial cells as well as activation of osteoblasts and vascular growth directed by PDGF and TGFs. It is evident radiographically that adding PDGF to graft material significantly reduces the time for graft consolidation and maturation and improves trabecular bone density.

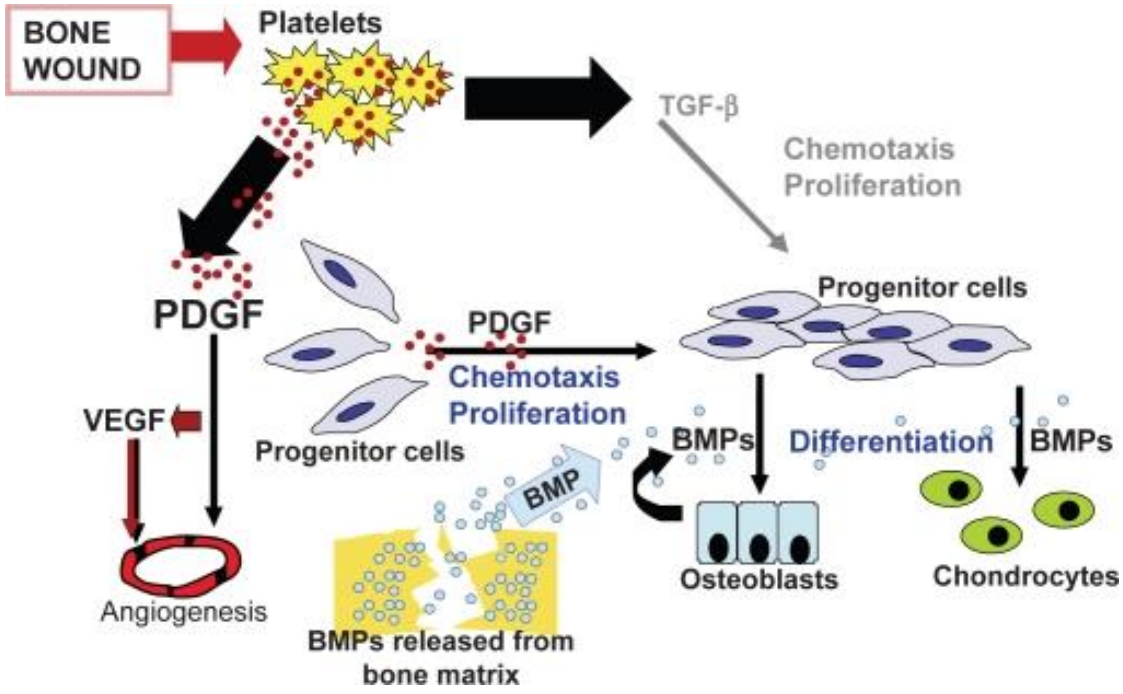


Figure 7: Platelet-derived growth factor (PDGF). Mechanism of action and bone regeneration- As a consequence of injury alpha granules containing PDGF are released by platelets initiate angiogenesis, chemotaxis, and mitogenesis. In addition PDGF upregulates vascular endothelial growth factor (VEGF) further enhancing angiogenesis. Transforming growth factor-beta (TGF-β) also plays a role in chemotaxis and cell proliferation during wound-healing. The attraction of osteoprogenitor cells (chemotaxis) and their increase in number (mitogenesis) provide a pool of osteo-regenerative cells that will respond to the bone morphogenetic proteins (BMPs). BMP is a differentiating factor. Consequently BMPs and PDGF are primary and powerful co-regulatory controls for healing and regeneration of bone.

Clinical Efficacy and Safety

Lynch and co-workers (1989) first published evidence of the regenerative potential of PDGF-BB when used to treat naturally occurring periodontal defects in dogs. This study showed increased cellular activity after treatment with PDGF-BB leading to increased bone, cementum and periodontal ligament regeneration. In a related study examining its use around dental implants direct application of an rhPDGF/IGF mixture into implant sites in dogs produced a two to three fold increase in the number of peri-implant spaces filled with bone at early time points. Promising results were also seen in immediate extraction socket implants treated with polytetrafluoroethylene

(PTFE) membranes and PDGF/IGF. Bone density and bone-to-implant contact were increased twofold for the growth factor treated sites compared to the membrane alone or membranes combined with bone grafts. These early studies established clinical evidence for the potential of PDGF treatment in periodontal and peri-implant sites.

The adjunctive effect of rhPDGF therapy for implant site development has also been investigated in animal models. Simion and co-workers (2006) conducted an animal study to investigate vertical bone augmentation following the application of rhPDGF-BB. Bilateral severe bone defects were surgically created in the mandibles of foxhound dogs. Three months later each of the defects was treated following one of three augmentation protocols: 1. Anorganic bovine bone blocks covered with a collagen membrane, 2. Anorganic bovine bone blocks infused with rhPDGF-BB, or 3. Anorganic bovine bone blocks infused with rhPDGF-BB covered with a collagen membrane. This study demonstrated that rhPDGF-BB infused matrix significantly enhanced bone formation and gingival healing in large, critical size alveolar bone defects. Radiographic and histological analysis indicated that the greatest bone regeneration occurred for the rhPDGF-BB infused graft block without the collagen membrane.

Studies by Schwarz and coworkers (2009) evaluated early healing outcomes following horizontal ridge augmentation. Bilateral mandibular surgically created defects were treated with either beta-tricalcium phosphate (β−TCP) covered with a collagen membrane (CM) or a combination of β−TCP and CM plus rhPDGF-BB following a split-mouth design. The group containing rhPDGF-BB showed increased mineralized tissue and total augmented area at 3 weeks. Taken together the promising clinical evidence of PDGF therapy in periodontal, peri-implant, and bone augmentation established the foundation for therapeutic use of PDGF in clinical applications.

The use of rhPDGF for dental implant site development (i.e. sinus augmentation), periodontal regeneration, horizontal bone augmentation and ridge preservation has been investigated in human studies.

An early human clinical trial by Howell and co-workers (1997) evaluated rhPDGF/IGF treatment applied to osseous periodontal defects. The experimental sites received direct application of growth factors contained in a methylcellulose matrix to improve retention. A statistically significant increase in alveolar bone formation occured in the growth factor treated sites at nine months post-operatively compared to untreated control sites. Average bone height for the PDGF/IGF group was 2.08 mm and 43.2 % osseous defect fill was achieved compared to 0.75 mm new bone height and 18.5% fill in the control sites.

Based on the principles of tissue engineering the use of growth factor enhanced matrix consisting of rhPDGF-BB in combination with an osteoconductive scaffold (i.e., autograft, allograft, xenograft, or a synthetic matrix, such as beta-TCP) was proposed for periodontal regeneration. The rationale underlying this approach is that PDGF stimulates angiogenesis, promotes cell migration into the bone defect from the surrounding tissue margins and upregulates cell proliferation. The matrix, in addition to its role as a growth factor delivery vehicle, provides mechanical support for migrating cells and contributes to the formation of new bone, cementum and/or periodontal ligament.

The regenerative effects of rhPDGF in combination with mineralized freeze dried bone allograft have been documented clinically in a case study by Nevins and co-workers (2003). Two patients presenting with severe bone loss (teeth with a poor to hopeless prognosis) and requiring surgical bone grafting were treated with rhPDGF-BB (concentration of 0.3 (Case 1) or 1.0 mg/mL (Case 2)) enhanced mineralized allograft. The growth factor-enhanced matrix was packed into the

defect and an absorbable barrier membrane was placed over the defect prior to soft tissue closure. At six months probing depths for both patients were 3 mm and gingival recession was 0 mm and 3 mm for Case 1 and Case 2, respectively. The gains in clinical attachment level relative to baseline were 7 mm and 2 mm for Case 1 and Case 2, respectively. No adverse effects associated with either rhPDGF-BB dose were observed. These results demonstrate that rhPDGF-BB combined with freeze-dried bone allograft provides an effective treatment for severe periodontal bone loss.

An alternative to an allograft is a completely synthetic growth factor enhanced matrix. Recombinant rhPDGF-BB has been combined with β−tricalcium phosphate (β-TCP) a well-established resorbable ceramic biomaterial commonly used in oral reconstructive surgery. The results of a large, multicenter clinical trial evaluating the effectiveness of rhPDGF-BB combined with a porous β-TCP matrix have been recently reported by Nevins and co-workers (2005). This study included 180 participants with one interproximal periodontal defect 4 mm or deeper after debridement. Three treatment groups were evaluated: 1) β-TCP plus 0.3 mg/ml rhPDGF-BB (Group I), 2) β-TCP plus 1.0 mg/ml rhPDGF-BB (Group II), and 3) β-TCP plus buffer alone (Group III). Defects were classified as 1-wall, 2-wall or 3-wall / circumferential indicating the extent of involvement and severity. At the time of surgery β-TCP granules were saturated with rhPDGF-BB before the graft was placed in the defect site. Excellent healing was observed for all defects treated with rhPDGF-BB. This study demonstrated that there was a significantly greater clinical attachment level gain at three months for the 0.3 mg/ml rhPDGF-BB (Group I) compared to the β-TCP controls (Group III) indicating an early benefit of rhPDGF-BB treatment. At six months the clinical attachment level gain for the lower rhPDGF-BB concentration group continued to be greater than the control group although statistical significance was not achieved. Additionally rhPDGF-BB treatment resulted in significantly less gingival recession at three months compared

to the untreated control group. This difference was no longer apparent at six months, however, as the control group exhibited a slight gain in gingival height over time. Increasing the rhPDGF-BB concentration appeared to reduce the effectiveness of the growth factor-enhanced matrix. No statistically significant differences were observed in clinical attachment level or gingival recession for the higher rhPDGF-BB concentration (Group II) compared to the β-TCP controls. Radiographic assessment revealed that bone fill was significantly increased at six months for the lower rhPDGF-BB concentration compared to both the higher rhPDGF-BB concentration and the control group. A subgroup analysis further indicated that rhPDGF-BB treatment improved bone fill in smokers for all defect types (1, 2, 3 wall and circumferential). Similarly linear bone growth was also significantly greater for Group I compared to Groups II and III. Placing these clinical results in perspective the use of 0.3 mg/ml rhPDGF and β-TCP for the treatment of periodontal defects compares very favorably to existing FDA approved treatments in terms of clinical attachment level gain and bone fill. This therapeutic approach significantly improved both clinical soft tissue and radiographic measures compared to the control group.

Recombinant rhPDGF has also been examined as a treatment for soft tissue recession defects. In a case series study conducted by McGuire and colleagues (2006) seven subjects presenting contralateral > 3 mm-deep recession defects (Miller Class I and II) received two different types of treatment in a split-mouth design. Test therapy consisted of a combination of rhPDGF/β-TCP and a collagen membrane. The control treatment applied was the gold standard for root coverage: the subepithelial connective tissue graft (CTG). Healing was evaluated at 8, 16, and 24 weeks following the interventions. Primary outcome measurement was recession depth. There was a favorable tissue response to the test therapy with clinical outcomes comparable to CTG in terms of root coverage and keratinized tissue width.

In a later publication McGuire and co-workers (2009) reported histologic and microtomographic analysis of human en-block samples of teeth that received either CTG or a combination of rhPDGF/β-TCP and a wound healing dressing. Two patients requiring the extraction of a total of six premolars as part of orthodontic therapy were included in the study. Gingival recession defects were surgically created in the buccal aspect of these teeth and when necessary, alveolar bone was resected to position the crest at 2–3 mm from the newly created gingival margin. In all teeth reference notches were created at the level of the new gingival margin and at the bone crest. Defects were left untouched for two months prior to root coverage procedures. A total of two CTG and four rhPDGF/β-TCP procedures were performed. After a nine-month healing period biopsies were obtained and defects were grafted. Clinically 100% root coverage was achieved in all surgical sites. However, histologic and microtomographic results were distinct for both treatment groups. While none of the CTG-treated sites showed signs of periodontal regeneration (healing characterized by a long juctional epithelium and parallel connective tissue fibers with minimal new cementum formation) all four rhPDGF/β-TCP-treated sites exhibited periodontal regeneration. This was shown by the presence of periodontal ligament interposed between newly formed cementum and alveolar bone situated above the reference apical notch.

Aim and Goals

Alveolar ridge augmentation is essential for successful implant therapy and depends on the biological performance of bone graft materials. This study will help to determine healing capabilities and limitations of currently available bone substitutes combined with biological growth factors (PDGF and BMP).

The study used neonatal mouse calvarial bone organ cultures stimulated with ascorbate (vitamin C) as an antioxidant/reducing agent to determine the effect of PDGF and BMP on osteoblastic activity and new bone formation.

The aims of this study were as follows -

1. To evaluate the effect of PDGF on new bone formation in mouse calvarial organ cultures.
2. To evaluate the effect of GEM-21 (PDGF + B-TCP) on new bone formation in mouse calvarial organ cultures.
3. To evaluate the effect of BMP-2 on new bone formation in mouse calvarial organ cultures.
4. To compare the effect of PDGF, GEM-21 and BMP-2 on osteoblastic activity.

MATERIALS & METHODS

Calvaria from 5-7 day neonatal CD-1 mice (Charles River Laboratories, MA) were dissected under sterile conditions. Calvaria were cut in the occipital lobe and partially in the frontal lobe to produce a trapezoid structure and rinsed in culture medium. The live bone organ culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with bovine serum albumin (BSA), 100 U/ml penicillin and 100 mg/ml streptomycin, 250 ng/ml amphotericin B (Gibco, Grand Island, NY) and ascorbic acid but no fetal calf serum (FCS). The culture media was supplemented with 1 nM sodium ascorbate (Sigma Co.) as it acts as an antioxidant/reducing agent and is an essential biomolecule during collagen biosynthesis and hence, formation and maintenance of bone. The culture media was thus modified to mimic the clinical scenario.

Following groups and number of calvaria per group were set up:

Experiment I

(3 weeks – 21 days)

(a) Control - critical bone defect (2); non critical bone defect (2)

(b) Freeze dried bone allograft (FDBA)

(c) Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF)

(d) B- Tricalcium phosphate (B-TCP) + Platelet derived growth factor (PDGF) [GEM-21]

(e) Freeze dried bone allograft (FDBA) + B-Tricalcium phosphate (B-TCP) + Platelet derived growth factor (PDGF)

Experiment II

A. 3 weeks – 21 days

(a) Collagen + Bone Morphogenetic Protein 2 (BMP-2)

(b) Collagen + Freeze dried bone allograft (FDBA) + Bone Morphogenetic Protein 2 (BMP-2)

B. 1 week – 7 days

(a) Collagen + Freeze dried bone allograft (FDBA)

(b) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF)

(c) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) + B- Tricalcium phosphate (B-TCP)

(d) Collagen + Freeze dried bone (FDBA) + Bone Morphogenetic Protein 2 (BMP-2)

(e) Collagen + Bone Morphogenetic Protein 2 (BMP-2)

The calvaria were placed on stainless steel grids so that the bone was elevated and the media formed a thin film over the periosteal surface. They were incubated at 37°C with 5 % CO2 in a tissue culture incubator (NAPCO, Winchester, VA) for 21 days. Collagen was used as a carrier (figure 8). The media was changed every 2 days and the spent media were stored at 4°C for calcium analysis of levels. At the end of the experiment, two calvaria from each treated group were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H & E) for histological observation. The two calvaria from each treated group were used for neutral red (NR) staining.

Study Parameters

Quantitative analysis of the changes in media calcium in mouse calvarial organ cultures

Media calcium levels were determined by an ArsenazoI II microplate calcium assay. The assay reagent contained 300mg/l Arsenazo III in a 50mM sodium acetate buffer pH4.8 (Yingst and Hoffman, 1983). 10 µl from each sample, all time points, were assayed in duplicates with the addition of 190 µl of the Arsenazo III reagent in a 96 well plate and absorbance measured at 560nm. Calcium contents were calculated from a standard curve from 0 to 70 nmol [Ca2+].

Quantitative analysis of Alkaline Phosphatase activity for osteoblast quantification in mouse calvarial organ cultures

ALP activity, a marker for osteoblast and bone formation, was determined by release of the enzyme from the calvarial bone cellular layer. ALP activity was measured by incubation of 50 ml of cell extract in duplicates with 250 ml of 0.15 M NaCl +30 m M NaHCO3, pH8.0, in the presence of 16mM p-nitrophe- nyl phosphate(pNPP, SigmaCo.) for 30min at 37°C using a 96 well microplate. The absorbance at 405nm was measured and the rate of hydrolysis of the substrate and amount of p-nitrophenol (pNP) released was calculated using the extinction coefficient ε405 nm¼ 12,500mol−1 l cm−1 to calculate the concentration in moll−1 and the rate of hydrolysis using the incubation time in moll−1 min−1.

Neutral red (NR) staining to visualize osteoclast activity

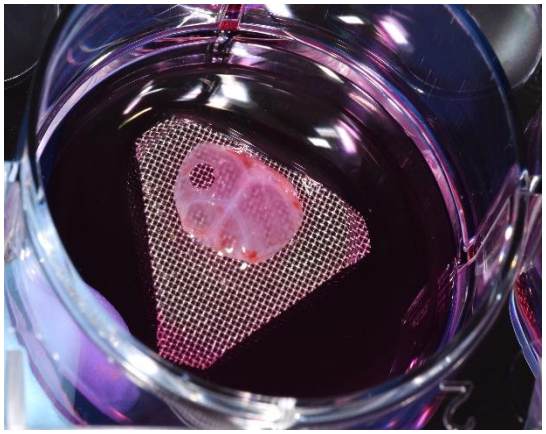
NR has been used for staining osteoclasts (Curtin et al., 2009, 2012; Sidgui et al., 1995; Braidman et al., 1990). They take up NR rapidly and their large size and multinucleated nature provide a clear microscopic contrast to other bone cells. At the end of the 21 day culture period

calvaria were incubated for 45 min at 37°C with NR, 70 µg/ ml (Sigma, Co.). The mature multinucleated osteoclasts were evaluated microscopically in all the experimental groups. Following this NR stained samples fixed in 10% formalin overnight.

Histological observations using H&E staining in mouse calvarial organ cultures

At the end of the 21 day culture period two calvaria from each treated group were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H & E) for histological observation.

(a) Calvaria with collagen tape



(b) Calvaria on steel grid placed in media

(c) GEM-21 being added to culture

Figure 8. Mouse Calvarial Bone Cultures

Statistical Analysis

Data was presented as means $\pm$SD. The paired Student's t-test (one-tail and two-tail) was used to compare groups. The differences between groups were considered statistically significant for $p<0.05$.

RESULTS

Experiment I

Quantitative analysis of the changes in media calcium in mouse calvarial organ cultures

The total change in media calcium was determined over the three-week culture period for all five groups. Fig.9 shows total cumulative changes in media calcium for the groups (a)–(e). Cultures treated with freeze dried bone alone showed maximal calcium release whereas those treated with GEM-21 showed minimal concentration of calcium in the media indicating enhanced osteoblastic activity and new bone formation. The results for calcium release were analyzed by paired Student's t-test to show statistical significance.

Quantitative analysis of the changes in media calcium in presence or absence of PDGF in mouse calvarial organ cultures

Cultures treated with PDGF showed statistically insignificant inhibition of calcium release when compared with controls and those treated with freeze dried bone alone. Addition of B-TCP to PDGF and FDBA did not show much effect on calcium release. However, B-TCP with PDGF in absence of FDBA showed maximum osteoblastic activity.

Quantitative analysis of the changes in media calcium in presence or absence of FDB in mouse calvarial organ cultures

Cultures treated with FDBA did not show any significant effect on calcium release in either of the four groups- a) controls, b) those treated with FDBA alone, c) those with FDBA and PDGF or d) those with FDBA and GEM-21 whereas cultures treated with GEM-21 in the absence of FDBA

showed minimal calcium release. This study suggests that GEM-21 alone stimulates the osteoblastic activity in mouse calvarial bone organ cultures and FDBA alone has a stimulatory effect on osteoclastic activity.

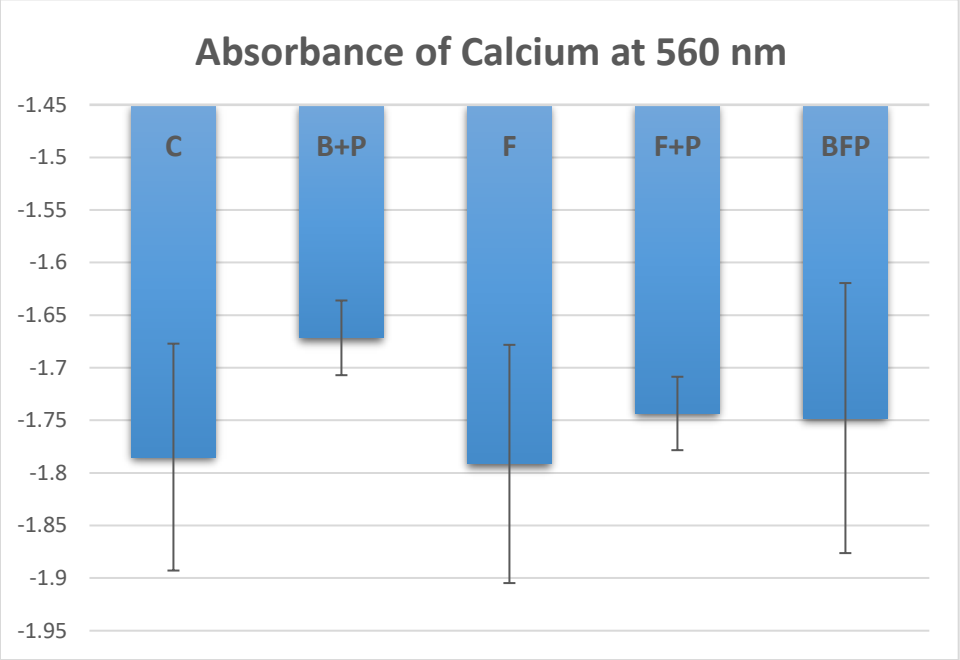


Figure 9: Quantitative analysis of the changes media calcium in mouse calvarial organ cultures over a three-week period. Calcium release is shown for the five groups- –(a) control-**C**; (b) B- Tricalcium phosphate (B-TCP) + Platelet derived growth factor (PDGF) [GEM-21] - **B+P**; (c) freeze dried bone allograft (FDBA) - **F**; (d) freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) – **F+P** and (e) Freeze dried bone allograft (FDBA) + B-Tricalcium phosphate (B-TCP) + Platelet derived growth factor (PDGF) - **BFP**. The study shows that GEM-21 inhibits calcium release indicating higher osteoblastic activity. Addition of FDBA alone to the media does not significantly effect calcium release when compared to control. Groups (d) and (e) treated with FDBA+PDGF and FDBA+GEM-21 respectively showed similar decrease in calcium release into the media indicating enhanced osteoblastic activity but to a lesser degree when compared with GEM-21 alone (Group b).

Quantitative analysis of ALP enzyme activity for osteoblast quantification in mouse calvarial bone formation model

Quantitative ALP activity is a measure of osteoblast numbers in calvarias cultured under different conditions as indicated in Fig.10. ALP activity was determined over the three week culture period for the five groups. The results of ALP activity are consistent with observed calcium release levels shown above. Cultures treated with GEM-21 alone showed significantly increased ALP activity indicating higher number of osteoblasts. However, this activity was inhibited in the presence of FDBA and PDGF. Cultures treated with FDBA alone and those with FDBA in combination of PDGF showed slightly higher ALP activity when compared to control**s**. Cultures treated with both FDBA and GEM-21 showed ALP activity lower than that of control**s** suggesting inhibition of osteoblas**t**ic multiplication. However, these results were statistically insignificant.

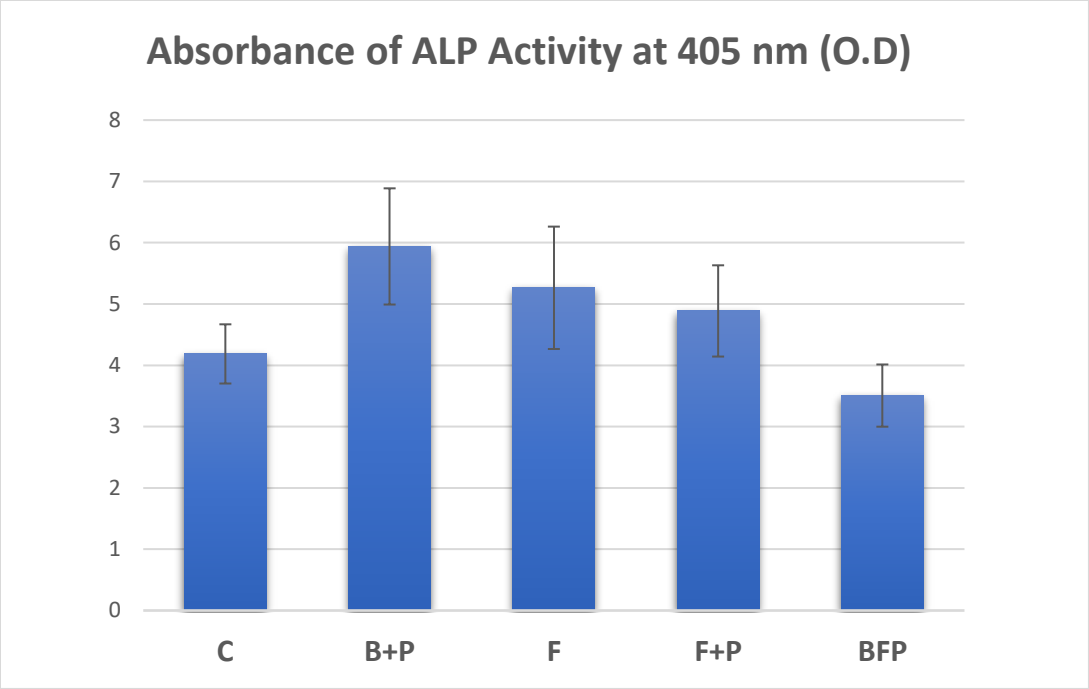


Figure 10: Quantitative analysis of ALP enzyme activity in mouse calvarial bone formation model over a period of three weeks. ALP enyme activity is ahown for the five groups- (a) control- **C**; (b) B- Tricalcium phosphate (B-TCP) + Platelet derived growth factor (PDGF) [GEM-21] - **B+P**; (c) freeze dried bone allograft (FDBA) - **F**; (d) freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) – **F+P** and (e) Freeze dried bone allograft (FDBA) + B-Tricalcium phosphate (B-TCP) + Platelet derived growth factor (PDGF) - **BFP**. Cultures treated with GEM-21 alone (group b) show significantly increased ALP activity indicating higher number of osteoblasts. This activity was inhibited in the presence of FDBA and PDGF as seen for groups (c), (d) and (e). Cultures treated with FDBA alone and those with FDBA in combination of PDGF showed slightly higher ALP activity when compared to control**s**. Group (e) treated with both FDBA and GEM-21 showed the least ALP activity. These results are consistent with the results of quantitative analysis of the changes in media calcium (Fig.9).

Visualization of osteoclast formation by neutral red in mouse calvarial organ cultures

This study visualized osteoclasts by neutral red staining to gain further insights into the biologic events and confirm the results of quantatitive analyses. Fig.11 shows NR staining of calvaria treated with FDBA alone, FDBA and PDGF, FDBA and GEM-21 and GEM-21 alone respectively. Cultures treated with GEM-21 did not show multinucleated osteoclasts whereas those treated with freeze dried bone alone show numerous NR stained osteoclasts.

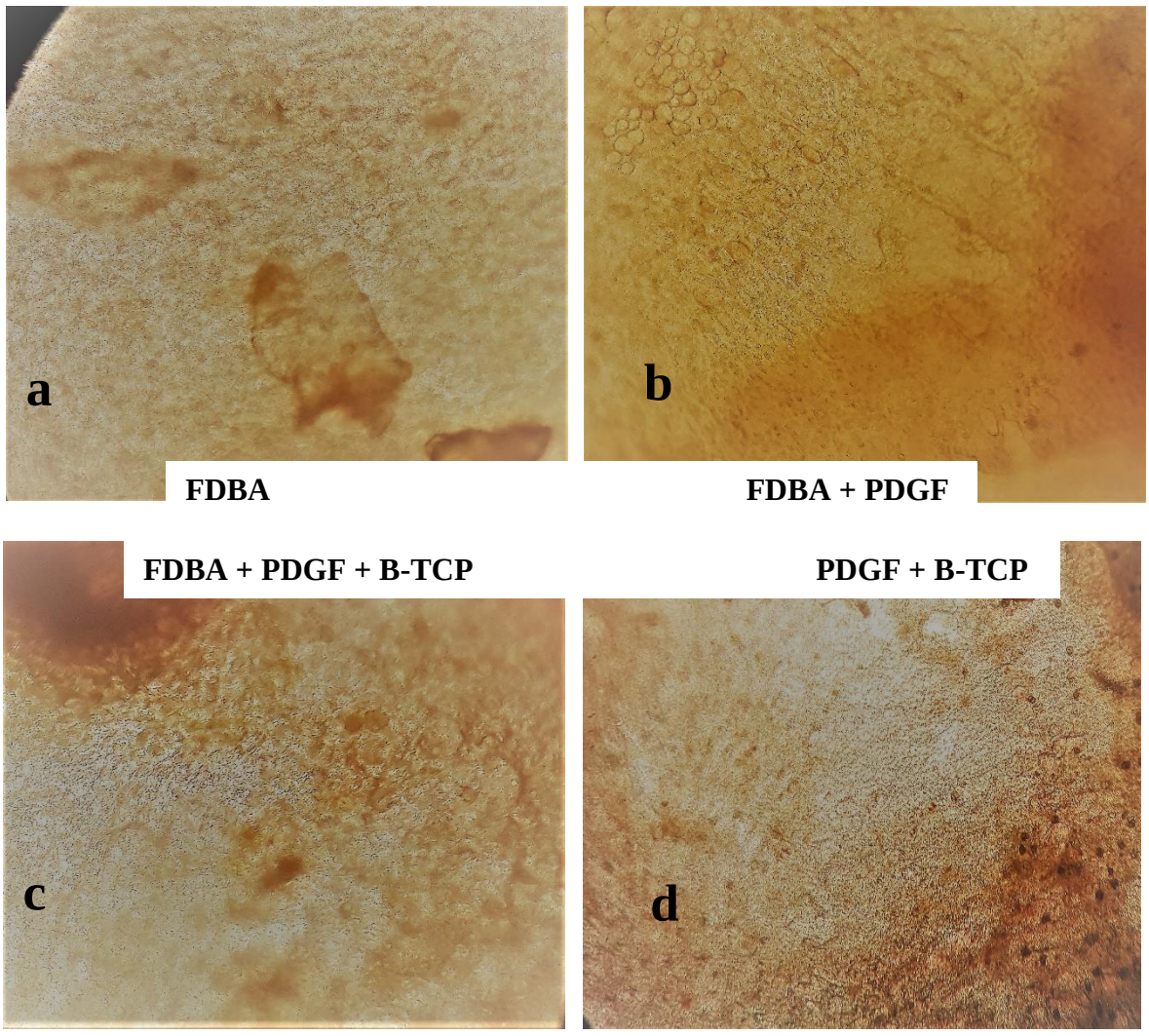
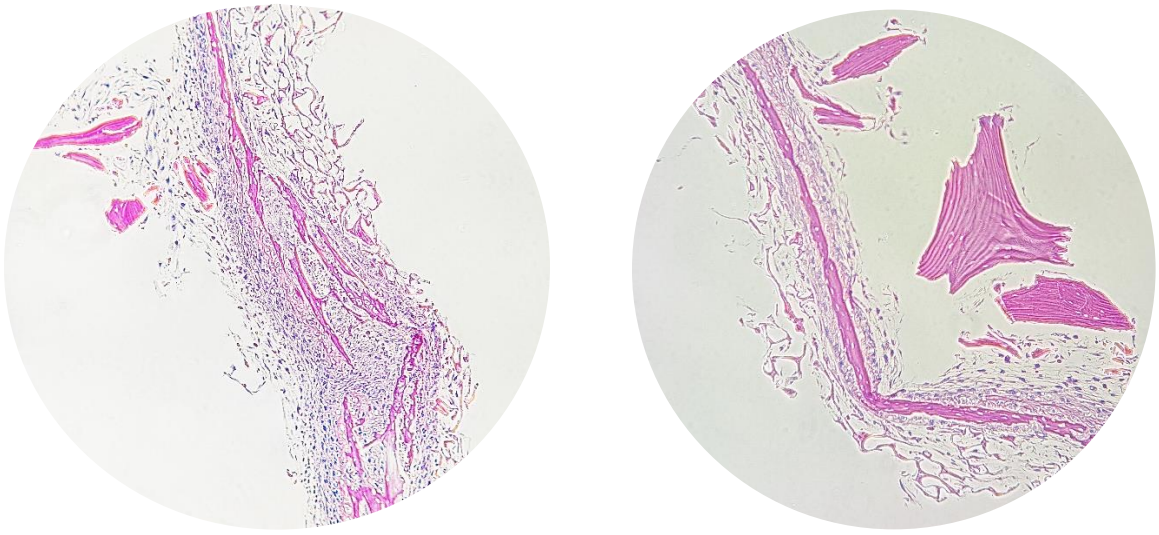


Figure 11: Visualization of osteoclast formation by neutral red staining in mouse calvarial cultures.

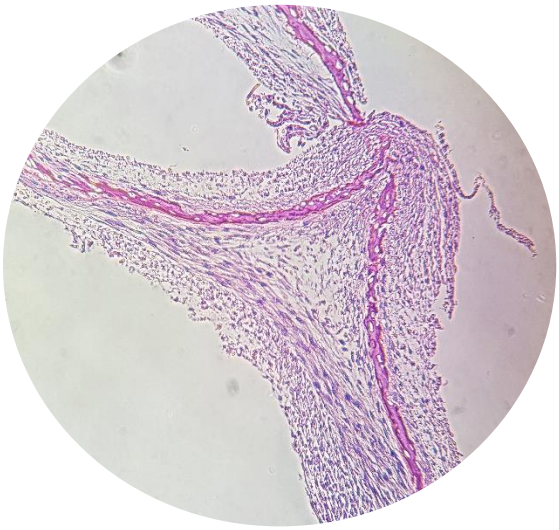
Histological observations using H&E staining in mouse calvarial organ cultures

The H&E stained sections of calvaria are illustrated in Figures 12-13. Figure 11 showed minimal osteoblast differentiation and osteoid formation for controls. Figure 13(a) showed significant osteoblast differentiation and new osteoid formation when cultures were treated with GEM-21 alone while figure 13(b) showed osteoclastic resorption in presence of FDBA. Figures 13(c) and (d) showed osteoblastic differentiation and some amount of new bone formation in presence of FDBA + PDGF and FDBA + GEM-21 respectively.

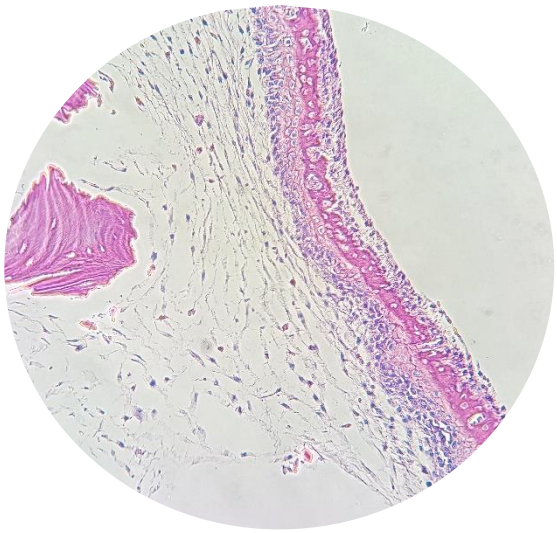


Control – Non-critical **Control- Critical**

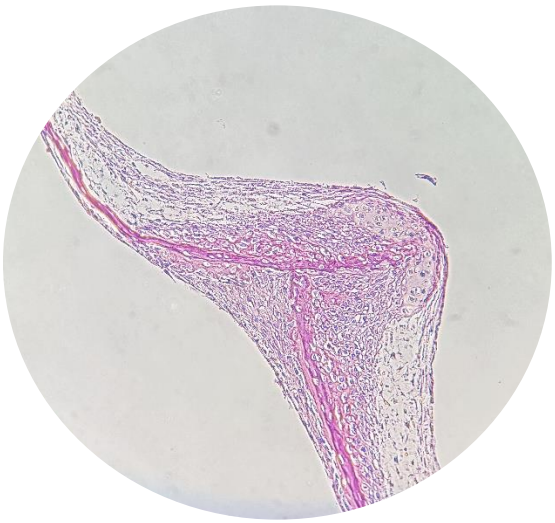
Figure 12. Microscopic observation of mouse calvarial organ cultures using H&E stain – control groups



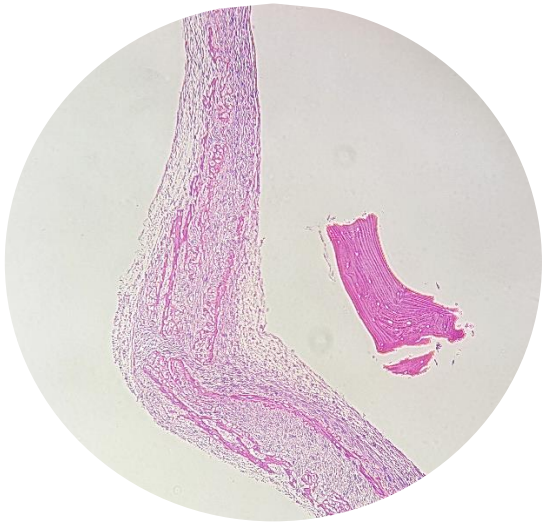
(a) GEM-21



(b) FDBA



(c) FDBA+ PDGF



(d) FDBA+ GEM-21

Figure13. Microscopic observation of mouse calvarial organ cultures using H&E stain – experimental groups

Experiment II

Quantitative analysis of the changes in media calcium in mouse calvarial organ cultures

Quantitative analysis of the changes in media calcium levels after 1 week

The total change in media calcium level was determined after one week for all five groups (Fig.14). All the differences were statistically significant when analyzed by paired Student's t-test. Freeze dried bone in combination with PDGF and B-TCP showed minimum calcium release when compared with freeze dried bone alone or in combination with PDGF or BMP.

BMP alone or in combination with freeze dried bone showed slightly lower calcium levels in the media when compared with control or freeze dried bone alone suggesting stimulatory effect of BMP on new bone formation. Interestingly PDGF with or without B-TCP showed higher stimulatory effect on osteoblastic activity than BMP.

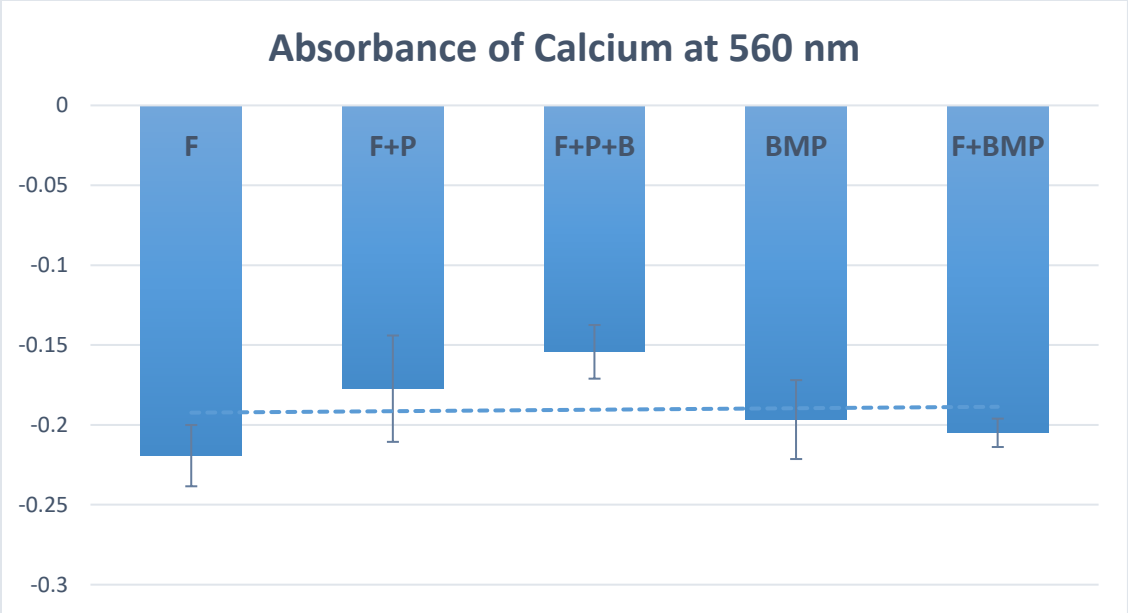


Figure 14: Quantitative analysis of the changes in media calcium in mouse calvarial organ cultures after 1 week is shown for the following groups – (a) Collagen + Freeze dried bone allograft (FDBA) - **F**; (b) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) – **F+P**; (c) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) + B- Tricalcium phosphate (B-TCP) – **F+P+B**; (d) Collagen + Bone Morphogenetic Protein 2 (BMP-2) – **BMP**; (e) Collagen + Freeze dried bone (FDBA) + Bone Morphogenetic Protein 2 (BMP-2) – **F+BMP.** Freeze dried bone in combination with PDGF and B-TCP (group c) shows minimum calcium release when compared with freeze dried bone alone (group a) or in combination with PDGF or BMP (group b or e). BMP alone (group d) or in combination with freeze dried bone (group e) shows slightly lower calcium level in the media when compared with freeze dried bone alone suggesting stimulatory effect of BMP on new bone formation. However, this effect is much less than that of freeze dried bone, PDGF and B-TCP combined (group c).

Quantitative analysis of the changes in media calcium levels after 3 weeks in presence or absence of FDBA

The total change in media calcium level for cultures treated with BMP-2 and collagen was determined after three weeks in the presence or absence of FDBA (Fig.15).

BMP alone in the absence of FDBA showed lower levels of calcium in the media suggesting enhanced osteoblatic activity and new bone formation. This result was statistically significant when analysed using student's t -test. This result is consistent with the quantitative analysis of media calcium levels observed after one week (Fig.14).

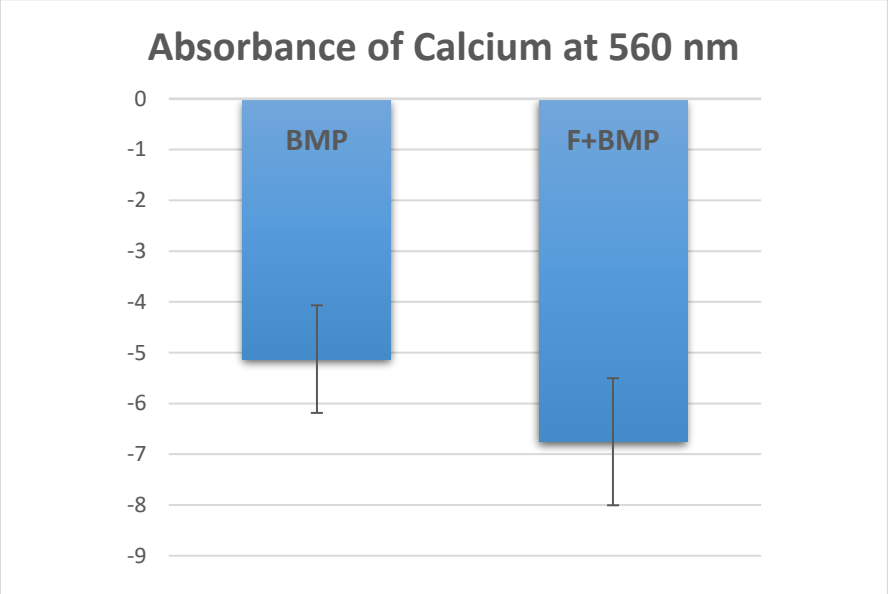


Figure 15: Quantitative analysis of the changes in media calcium levels after 3 weeks in presence or absence of FDBA. BMP alone in absence of FDBA showed lower levels of calcium in the media suggesting enhanced osteoblatic activity and new bone formation. Similar results were observed on quantitative analysis of media calcium levels after one week as shown above (Fig.14).

Quantitative analysis of ALP enzyme activity for osteoblast quantification in mouse calvarial bone formation model

Quantitative analysis of ALP enzyme activity in mouse calvarial bone formation model after 1 week

ALP enzyme activity was determined after one week for the following groups – (a) Collagen + Freeze dried bone allograft (FDBA); (b) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF); (c) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) + B- Tricalcium phosphate (B-TCP); (d) Collagen + Bone Morphogenetic Protein 2 (BMP-2); and (e) Collagen + Freeze dried bone (FDBA) + Bone Morphogenetic Protein 2 (BMP-2) (Fig.16).

BMP alone showed highest ALP activity indicating highest number of osteoblasts thus, enhanced bone formation. FDBA in combination with BMP had a similar effect whereas FDBA with PDGF showed minimum ALP activity. FDBA alone or with GEM-21 showed slightly lower ALP activity than that with BMP. These results are consistent with the results of quantitative analysis of the changes in media calcium levels observed after 1 week (Fig.14).

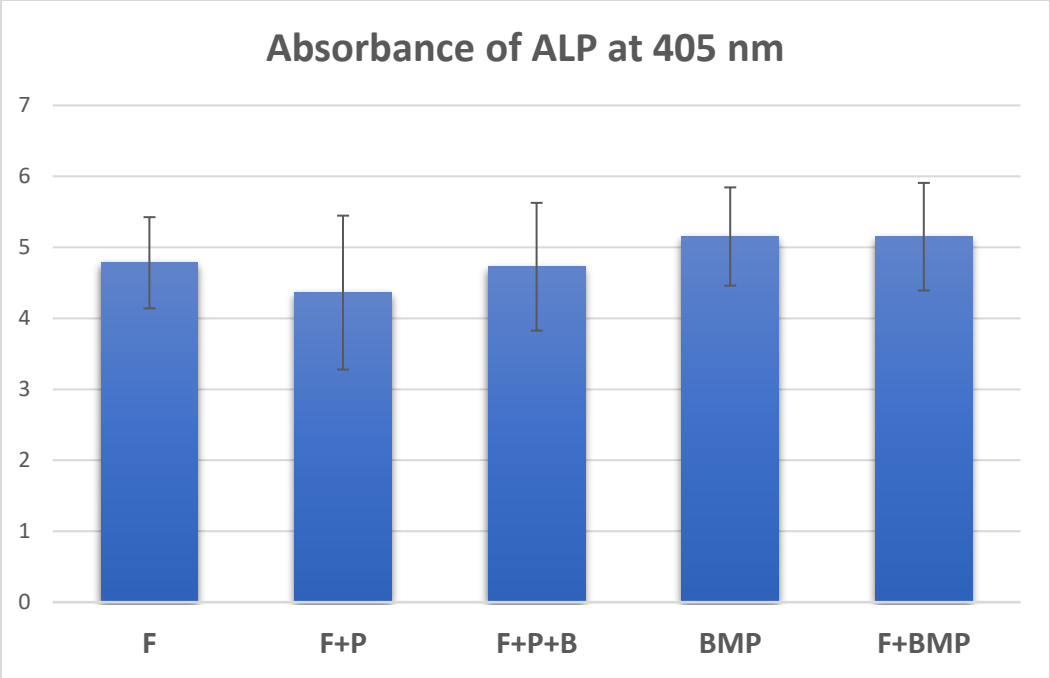


Figure 16: Quantitative analysis of ALP activity in mouse calvarial bone formation model after 1 week for the following groups – (a) Collagen + Freeze dried bone allograft (FDBA) - **F**; (b) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) – **F+P**; (c) Collagen + Freeze dried bone allograft (FDBA) + Platelet derived growth factor (PDGF) + B- Tricalcium phosphate (B-TCP) – **F+P+B**; (d) Collagen + Bone Morphogenetic Protein 2 (**BMP**-2); and (e) Collagen + Freeze dried bone (FDBA) + Bone Morphogenetic Protein 2 (BMP-2) – **F+BMP**. BMP alone showed highest ALP activity indicating highest number of osteoblasts. FDBA in combination with BMP had a similar effect on osteoblasts. FDBA with PDGF showed minimum ALP activity but FDBA alone or with GEM-21 showed slightly higher ALP activity. It is important to note that BMP has more positive effect on ALP activity than FDBA alone or in combination with PDGF.

Quantitative analysis of ALP enzyme activity in mouse calvarial bone formation model after 3 weeks

Fig.17 depicts the ALP activity for cultures treated with BMP-2 and collagen in the absence or presence of FDBA after three weeks of incubation.

BMP alone in the absence of FDBA showed higher ALP activity indicating higher osteoblast multiplication. Thus, FDBA inhibits the effect of BMP on osteoblasts. This result was statistically significant when analysed using student's t -test and supported by the changes in media calcium levels observed after three weeks (Fig.15).

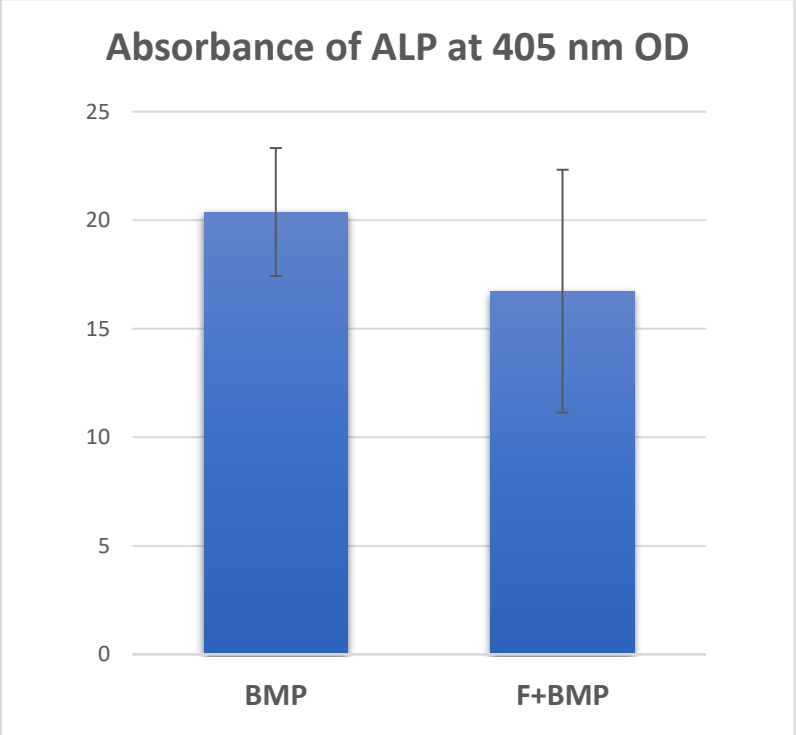
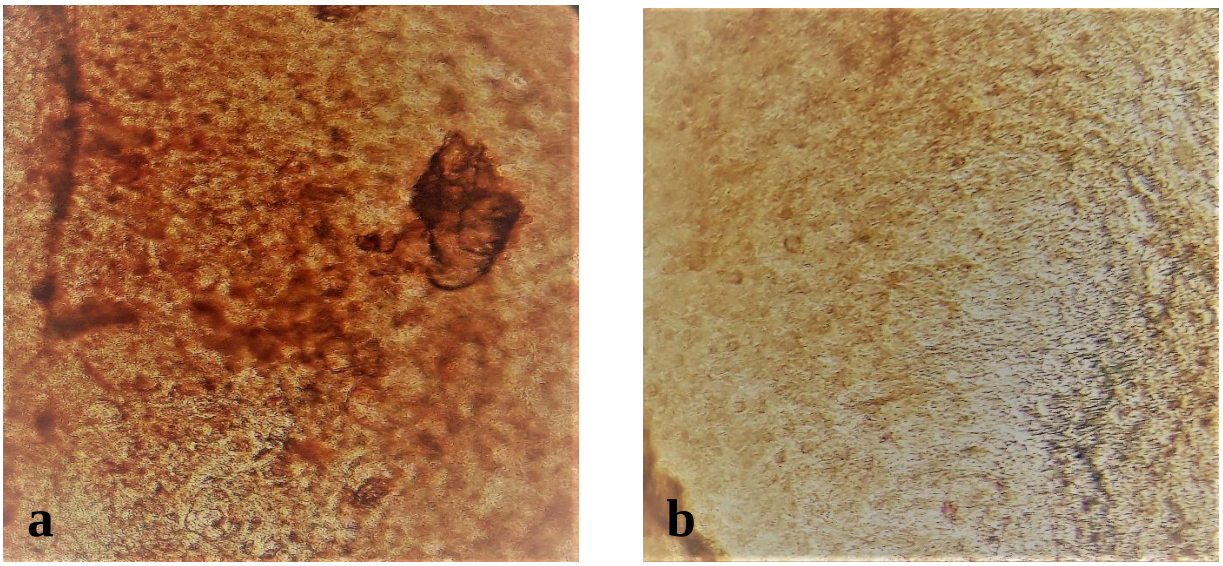


Figure 17: Quantitative analysis of ALP enzyme activity in mouse calvarial bone formation model after 3 weeks for two groups treated with BMP-2 with or without FDBA. As seen above, BMP alone in the absence of FDBA showed higher ALP activity. These results are consistent with the results of quantitative analysis of the changes in media calcium levels observed after 3 weeks (Fig.15).

Visualization of osteoclast formation by neutral red in mouse calvarial organ cultures after 1 week

Fig.18 depicts the NR stained osteoclasts for cultures treated with BMP-2 and collagen in the absence or presence of FDBA after one week of incubation. Cultures treated with BMP alone did not show multinucleated osteoclasts whereas those treated with BMP in addition to freeze dried bone show a few NR stained osteoclasts. This indicates an inhibitory effect of BMP on osteoclastic activity and correlates with the quantitative calcium analysis above.

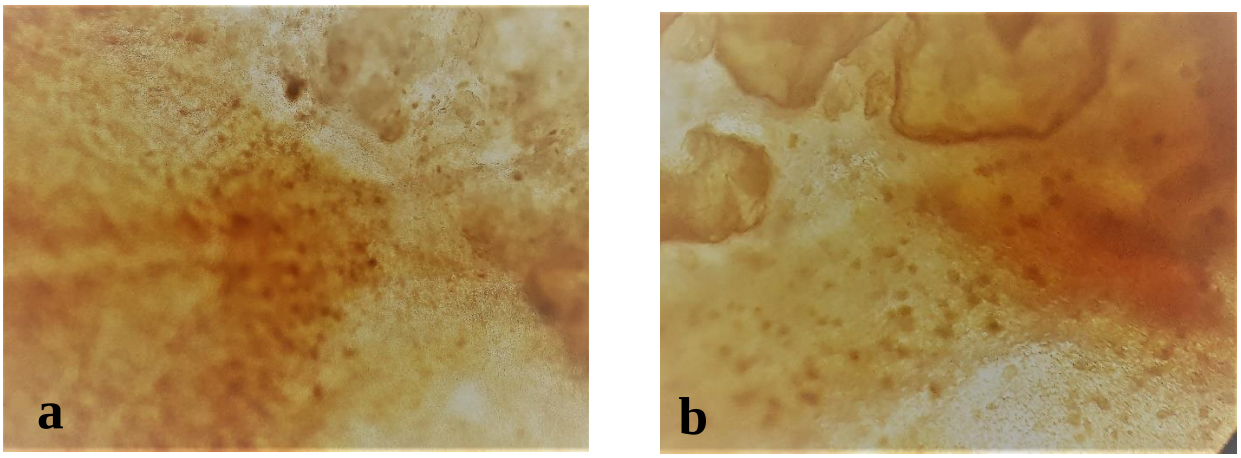


BMP alone **BMP + FDBA**

Figure 18: Visualization of osteoclast formation by neutral red staining in mouse calvarial cultures treated with BMP in absence or presence of FDBA after one week.

Visualization of osteoclast formation by neutral red in mouse calvarial organ cultures after 3 weeks

Fig.19 depicts the NR stained osteoclasts for cultures treated with BMP-2 and collagen in the absence or presence of FDBA after three weeks of incubation. Cultures treated with BMP alone and those treated with BMP in combination with freeze dried bone did not show NR stained multinucleated osteoclasts. This indicates new bone formation in both the cultures after three weeks.

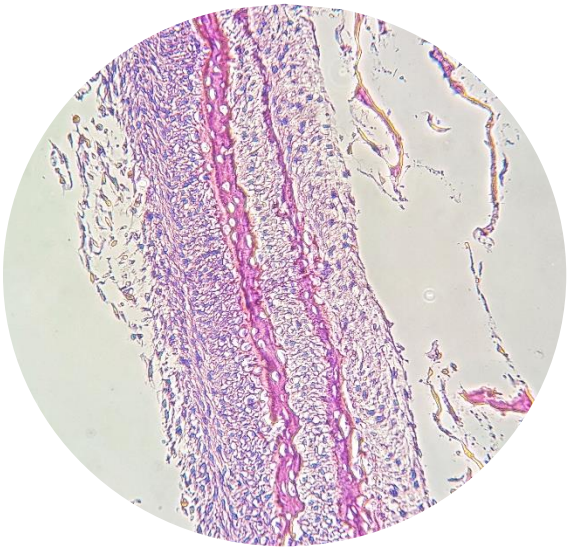


BMP alone **BMP + FDBA**

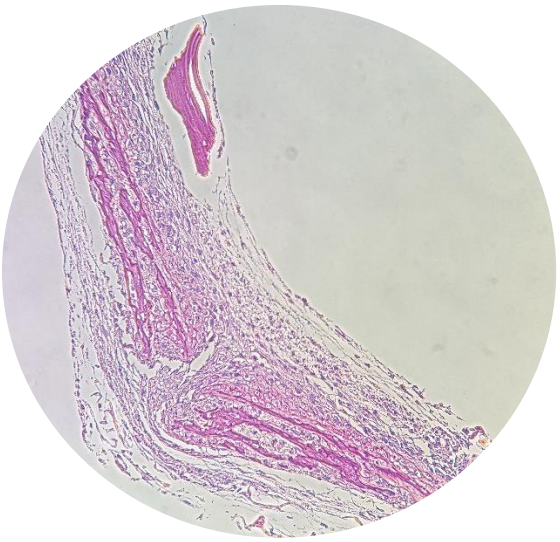
Figure 19: Visualization of osteoclast formation by neutral red staining in mouse calvarial cultures treated with BMP in absence or presence of FDBA after three weeks. Note the absence of multinucleated osteoclasts in both the cultures.

Histological observations using H&E staining in mouse calvarial organ cultures treated with BMP

The H&E stained sections of calvaria treated with BMP in presence and absence of FDBA are illustrated in Figure 20. Cultures treated with BMP alone showed marked osteoblastic differentiation and new osteoid formation. No osteoclastic resorption was seen whereas calvaria co-cultured with BMP and FDBA showed lower osteoblastic differentiation, thus FDBA inhibits the effect of BMP on new bone formation.



BMP +FDBA



BMP alone

Figure 20. Microscopic observation of mouse calvarial organ cultures using H&E stain treated with BMP in absence and presence of FDBA at 3 weeks

DISCUSSION

It is well recognized that bone grafts and a number of bone substitutes assist in the regeneration of lost bone in the orofacial region. They induce bone formation through cellular signaling or through the transfer of osteocompetent cells or may simply provide a scaffolding and have a space maintaining function for the host to grow new bone. Over the last few decades there has been a focused effort to understand how growth factors influence regeneration of bone. The two growth factors approved by FDA for bone regeneration are PDGF and BMP-2. Lack of data demonstrating three-dimensional bone regeneration using PDGF or BMP with a xenograft inspired us to conduct this study. The study was conducted in two phases in which PDGF and BMP-2 were tested for efficacy in new bone formation.

This is the first study to compare the effect of PDGF to that of BMP-2 in vitro.

Collagen membrane was used as a carrier or delivery agent for the growth factors. It is easy to mould, adapts well to the bone defect and helps to keep the factors in contact with the bone. It also acts as a potent osteoinductive agent while at the same time providing a scaffold for newly mineralized tissue growth.

Non- critical defect measuring 1 mm in size and critical defect measuring 2 mm acted as controls in the study. The critical defect was created for all the experimental groups. The non-critical defect was expected to heal without any need for bone grafts or substitutes, which was observed after three weeks.

Cultures were treated with PDGF in combination with an osteoconductive scaffold. The rationale underlying this approach is that PDGF stimulates angiogenesis, promotes cell migration into the bone defect from the surrounding tissue margins and upregulates cell proliferation. The

scaffold, in addition to its role as a growth factor delivery vehicle, provides mechanical support for migrating cells and contributes to the formation of new bone. It has also been reported that PDGF in animal models may have either a dose dependent negative effect on bone formation by negating the actions of growth factors such as osteogenins and reducing graft resorption or be of insignificant added value. The varied responses reported show the importance of this study to evaluate two carriers- FDBA and B-TCP when used alone or in combination.

PDGF with B-TCP (GEM-21) showed highest new bone formation. PDGF is involved in the maturation and remodeling of newly formed blood vessels, angio- and vasculogenic cells might act as important targets initially responding to the application of this mitogenic factor. However, in vitro experiments in which cells are continuously exposed to PDGF for long periods, osteoblast differentiation is inhibited and proliferation is enhanced leading to an abundant number of cells but little mineralized matrix. When PDGF is used with B-TCP as scaffold an initial burst of PDGF was observed followed by limited availability of the growth factor at the site. This sustained release mechanism of GEM-21 explains its effectiveness in bone regeneration and is in agreement with the relative biologic activity assessed for the PDGF soak-loaded BCP observed as a statistically significantly increased mean ALP activity and lower calcium release into the media.

In contrast to our study, Ridgeway et al recently showed that residual particles of bio ceramic carrier when used with PDGF inhibit a robust regenerative response initiated by PDGF. The use of B-TCP as carrier is thus inhibitory for new bone formation. Further investigations are required to clearly understand the mechanism.

The rationale for using PDGF with FDBA was that PDGF is a potent mitogen (stimulator of cell proliferation) and chemotactic (causes directed cell migration) protein for alveolar bone

cells and improves angiogenesis (new blood vessel formation) while a bone graft offers a biological matrix conducive to cell growth and may contribute osteoinductive bone matrix proteins. In the presence of PDGF the graft also shows accelerated remodeling allowing for increased volume of new bone as seen in our study.

When comparing B-TCP with FDBA as a better scaffold B-TCP showed more promising results. Mcallister et al compared PDGF with B-TCP and bovine xenograft and reported 21% and 24% bone fill respectively, results in contrast to our study.

BMP is a mitogen stimulating the multiplication of osteoblasts and has the ability to transform mesenchymal stem cells into osteoprogenitor cells thus increased new bone formation observed as a statistically significantly increased mean ALP activity in experiment II. When used with collagen matrix it promotes direct membranous bone formation without an endochondral precursor. We observed that BMP induced bone formation has large amounts of bone marrow. The concomitant development of BMP-induced bone growth with its expanded network of bone marrow ensures that both the availability of sufficient numbers of dividing stem cells and the nutritional demands for rapid new bone formation are met. The findings of the histologic assessment in our study showed development of native bone through a de novo intramembranous pathway that replicates native bone development. Preosteoblast condensations were observed in association with blood vessels and osteoblasts were observed forming new bone trabeculae through appositional secretion of osteoid and mineralized matrix. Osteoclast remodeling of the trabeculae was also observed.

An interesting observation of our study was that the bone-forming response with BMP-2 was higher than that with PDGF. Since BMP is a differentiating factor its effect is more profound on bone regeneration. BMPs affect mesenchymal stem cell (MSC) differentiation;

however, PDGF has a more potent chemotactic and proliferation effect on MSCs. MSCs are influenced by BMP-2 to differentiate directly into osteoblasts for intramembranous bone development whereas PDGF enhances angiogenesis and proliferation. Park et al also demonstrated in vitro that PDGF suppresses osteogenic differentiation which might further explain its lower efficacy.

CONCLUSION

In conclusion this study proves that the incorporation of growth factors in an osteoconductive scaffold is a very promising treatment option. These growth and differentiation factors have a unique potential to induce new bone formation in mouse calvarial cultures a fact previously supported by compelling evidence from preclinical studies and clinical investigations.

This study was the first to compare BMP-2 and PDGF in vitro in which BMP-2 proved to be the most effective agent. Histological findings and biochemical parameters indicated osteoblastic differentiation and new bone formation. This establishes the background for further investigations and clinical trials to determine the efficacy, safety and applications of growth factors in regenerative dentistry.

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